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(54) Title: SECOND GENERATION MONOCLONAL ANTIBODIES HAVING BINDING SPECIFICITY TO TAG-72 AND HUMAN CARCINOMAS AND METHODS FOR EMPLOYING THE SAME (57) Abstract The present invention relates to second generation monoclonal antibodies having binding specificity to a tumor associated glycoprotein having an approximate molecular weight of $>10^6$ d ("TAG-72") and human carcinomas and methods for employing the same. Hybridomas producing such antibodies have been prepared.		

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1 SECOND GENERATION MONOCLONAL ANTIBODIES HAVING
2 BINDING SPECIFICITY TO TAG-72 AND HUMAN
3 CARCINOMAS AND METHODS FOR EMPLOYING
4 THE SAME

5 FIELD OF THE INVENTION

6 The present invention relates to second generation
7 monoclonal antibodies having binding specificity to a
8 tumor associated glycoprotein having an approximate
9 molecular weight of $>10^6$ d (hereinafter "TAG-72") and
10 human carcinomas, and methods for employing the same.

11 BACKGROUND OF THE INVENTION

12 Numerous monoclonal antibodies have been developed
13 which have binding specificity for a variety of human
14 carcinomas (see Schlom, et al., "Important Advances in
15 Oncology", Philadelphia, PA, J.B. Lippincott Co., Vol. 1,
16 pp. 170-192 (1984) and Schlom, Cancer Res., 46:3225-3238
17 (1986)). One of these monoclonal antibodies designated
18 B72.3 (see Colcher, et al., Proc. Natl. Acad. Sci. USA,
19 78:3199-3203 (1981) and U.S. Patents Nos. 4,522,918 and
20 4,612,282), is a murine IgG₁, and was developed using a
21 human breast carcinoma extract as the immunogen.
22 Monoclonal antibody B72.3 is produced by hybridoma B72.3
23 (ATCC No. HB-8108) and has been extensively studied.

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1 Monoclonal antibody B72.3 has been shown to be distinct
2 from other known monoclonal antibodies on the basis of:
3 (1) its binding specificity to TAG-72 (see Johnson, et
4 al., Cancer Res., 46:857-859 (1986)); (2) its binding
5 specificity to various types of human carcinoma tissues,
6 including breast, ovarian, lung, colorectal, endometrial,
7 and pancreatic carcinoma tissues (see Nuti, et al., Intl.
8 J. Cancer, 29:539-545 (1982); Stramignoni, et al., Intl.
9 J. Cancer, 31:543-552 (1982); Thor, et al., J. Natl.
10 Cancer Inst., 76:995-1006 (1986); and Thor, et al.,
11 Cancer Res., 46:3118-3124 (1986)); (3) its lack of
12 binding specificity to normal adult human tissues (see
13 Nuti, et al., Intl. J. Cancer, 29:539-545 (1982);
14 Stramignoni, et al., Intl. J. Cancer, 31:543-552 (1983);
15 Thor, et al., J. Natl. Cancer Inst., 76:995-1006 (1986);
16 and Thor, et al., Cancer Res., 46:3118-3124 (1986)); (4)
17 its ability to detect TAG-72 in serum (see Paterson, et
18 al., Intl. J. Cancer, 37:659-666 (1986) and Klug, et al.
19 Intl. J. Cancer, 38:661-669 (1986)); (5) its ability to
20 detect carcinoma cells in human effusions and fine needle
21 aspiration biopsies (see Szpak, et al., Acta Cytologica,
22 28:356-367 (1984); Johnston, et al., Cancer Res., 45:
23 1894-1900 (1986); Szpak, et al., Am. J. Path., 122:252-
24 260 (1986); Johnston, et al., Human Path., 17:501-513
25 (1986); Martin, et al., Am. J. Clin. Path., 86:10-18
26 (1986); Nuti, et al., Intl. J. Cancer, 37:493-498 (1986);
27 and Johnston, et al., Cancer Res., 46:6462-6470 (1986));
28 and (6) its binding specificity and prolonged binding to
29 human carcinomas both in experimental animal systems (see
30 Kennan, et al., J. Nucl. Med., 25:1197-1203 (1984) and
31 Colcher, et al., Cancer Res., 44:5744-5751 (1984)) and in
32 clinical trials (see Colcher, et al., Cancer Res., 47:
33 1185-1189 (1987) and Esteban, et al., Intl. J. Cancer,
34 39:50-58 (1987)).

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1 However, monoclonal antibody B72.3 is
2 disadvantageous in that (1) B72.3 does not have binding
3 specificity to every human carcinoma tissue of a
4 particular type, e.g., to every ovarian, colon carcinoma
5 tissue, etc. (see Nuti, et al., Intl. J. Cancer, 29:539-
6 545 (1982); Stramignoni, et al., Intl. J. Cancer, 31:543-
7 552 (1983); Thor, et al., J. Natl. Cancer Inst., 76:995-
8 1006 (1986); Thor, et al., Cancer Res., 46:3118-3124
9 (1986); and Hand, et al., Cancer Res., 43:728-735
10 (1983)); (2) B72.3 does not have binding specificity to
11 all carcinoma cells within a given human carcinoma mass
12 (see Nuti, et al., Intl. J. Cancer, 29:539-545 (1982);
13 Stramignoni, et al., Intl. J. Cancer, 31:543-552 (1983);
14 Thor, et al., J. Natl. Cancer Inst., 76:995-1006 (1986);
15 Thor, et al., Cancer Res., 46:3118-3124 (1986); and Hand,
16 et al., Cancer Res., 43:728-735 (1983)); (3) B72.3 does
17 not have binding specificity to most human carcinoma cell
18 lines in culture (see Hand, et al., Cancer Res., 43:728-
19 735 (1983); Hand, et al., Cancer Res., 45:833-840 (1985);
20 and Friedman, et al., Cancer Res., 45:5648-5655 (1985));
21 (4) it is difficult to obtain highly immunoreactive
22 F(ab')₂, F(ab') and F(ab) fragments from B72.3, such
23 fragments being necessary for efficient in vivo
24 immunodiagnostic and therapeutic applications; and (5)
25 since B72.3 is of the IgG₁ isotype, it is difficult to
26 conduct monoclonal antibody effector cell mediated
27 cytotoxicity or complement mediated cytotoxicity studies
28 using B72.3 (IgG_{2a}, IgG_{2b} or IgM isotypes being more
29 efficient for these applications).

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SUMMARY OF THE INVENTION

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Accordingly, an object of the present invention is to provide monoclonal antibodies which have binding specificity to a variety of human carcinomas, including human carcinomas of a given type for which B72.3 essentially has no binding specificity.

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Another object of the present invention is to provide monoclonal antibodies having high binding affinity for TAG-72 and human carcinomas.

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A further object of the present invention is to provide monoclonal antibodies from which highly immunoreactive F(ab')₂, F(ab') and F(ab) fragments can be easily obtained for use in in vivo immunodiagnosis and therapy of human carcinomas.

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A still further object of the present invention is to provide monoclonal antibodies from which recombinant antibodies can be obtained for use in in vivo immunodiagnosis and therapy of human carcinomas.

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An additional object of the present invention is to provide monoclonal antibodies of the IgG_{2a}, IgG_{2b} and IgM isotypes which have binding specificity for human carcinomas for use in conducting monoclonal antibody effector cell mediated cytotoxicity or complement mediated cytotoxicity studies.

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Still an additional object of the present invention is to provide methods for diagnosing in vitro and in vivo human carcinomas and methods for treating human carcinomas employing these monoclonal antibodies.

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Other objects and advantages of the present invention will become apparent from the Detailed Description of the Invention presented hereunder.

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The above and various other objects and advantages of the present invention are achieved by the second

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1 generation monoclonal antibodies of the present invention
2 which have binding affinity to both TAG-72 and to LS-174T
3 antigen(s).

4 Unless defined otherwise, all technical and
5 scientific terms used herein have the same meaning as
6 commonly understood by one of ordinary skill in the art
7 to which this invention belongs. Although any methods
8 and materials similar or equivalent to those described
9 herein can be used in the practice or testing of the
10 present invention, the preferred methods and materials
11 are now described. All publications mentioned hereunder
12 are incorporated herein by reference.

13 As used herein, the expression "second generation
14 monoclonal antibodies" means monoclonal antibodies
15 produced using, as the immunogen, an antigen which has
16 been affinity purified with a first generation monoclonal
17 antibody. As used herein, the expression "first
18 generation monoclonal antibody" means a monoclonal
19 antibody produced using, as the immunogen, a crude cell
20 extract.

21 The term "substantially" as used herein means
22 almost wholly or to a large extent, but not entirely.

23 LS-174T (ATCC No. CRL-188) is a variant of the
24 LS180 (ATCC No. CRL-187) colon adenocarcinoma line. It
25 is more easily subcultivated than the parent line. It
26 is tumorigenic in nude mice. The karyotype is similar
27 to that of LS180 with a missing X chromosome in a
28 majority of the cells. Electron microscopic studies
29 reveal abundant microvilli and intracytoplasmic mucin
30 vacuoles (see Tom, et al., In Vitro, 12:180-191 (1976)).

31 TAG-72 is an antigen found in the LS-174T tumor
32 cell line. Monoclonal antibody B72.3 binds to a high
33 molecular weight tumor associated glycoprotein identified
34 as TAG-72. Data has been presented as described in

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1 Johnson, et al., Cancer Res., 46:850-857 (1986), to
2 characterize the TAG-72 molecule as a mucin. This
3 conclusion is based on the following observations: (a)
4 TAG-72 has a high molecular weight ($>1 \times 10^6$) as shown by
5 its exclusion from a Sepharose CL-4B column; (b) the
6 density of TAG-72 determined by equilibrium
7 centrifugation in CsCl was 1.45 gm/ml, indicating a
8 heavily glycosylated glycoprotein; (c) TAG-72
9 demonstrates a change in migration after neuraminidase
10 digestion, indicating that it is a heavily sialylated
11 molecule with an abundance of O-glycosidically linked
12 oligosaccharides characteristic of mucins; (d) blood
13 group antigens commonly found on mucins are found on
14 affinity-purified TAG-72; and (e) chondroitinase ABC
15 digestion had no effect on TAG-72, thus demonstrating
16 that the TAG-72 epitope is not expressed on a chondroitin
17 sulfate proteoglycan.

18 More specifically, the above-described objects of
19 the present invention have been achieved by the second
20 generation monoclonal antibodies of the present
21 invention, immunoreactive fragments or recombinants
22 thereof which have binding specificity to TAG-72 and to
23 human carcinomas, including human carcinomas to which
24 antibody B72.3 has minimal binding specificity and with
25 minimal binding specificity to normal adult human
26 tissues. The term "minimal" means the least possible or
27 substantially inconsequential.

28 To another embodiment, the above-described objects
29 of the present invention have been achieved by a method
30 for diagnosing a human carcinoma or metastases thereof
31 comprising:

32 (a) obtaining a body sample, such as body
33 fluid, tissue or biopsy from a patient;

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1 (b) contacting the body sample material
2 with a second generation monoclonal antibody of the
3 present invention, an immunoreactive fragment or a
4 recombinant thereof;

5 (c) determining the level of binding of
6 second generation monoclonal antibody, immunoreactive
7 fragment or recombinant thereof to the body sample
8 material; and

9 (d) comparing the amount of second
10 generation monoclonal antibody, immunoreactive fragment
11 or recombinant thereof bound to substances present in the
12 body sample to a control sample or to a predetermined
13 base level, so that a binding greater than the control
14 level is indicative of the presence of human carcinomas
15 or metastases thereof.

16 In still another embodiment, the above-described
17 objects of the present invention have been achieved by a
18 method for diagnosing the presence of a human carcinoma
19 or metastases thereof comprising:

20 (a) administering to a patient a second
21 generation monoclonal antibody of the present invention,
22 an immunoreactive fragment or recombinant thereof,
23 conjugated to an imaging marker; and

24 (b) exposing the patient to a means for
25 detecting said imaging marker to identify areas of
26 imaging marker corresponding to a human carcinoma or
27 metastatic sites thereof in said patient.

28 In a still further embodiment, the above-described
29 objects of the present invention have been achieved by a
30 method of treating a patient afflicted with a human
31 carcinoma or metastases thereof, comprising administering
32 to a patient afflicted with carcinoma or metastases, a
33 pharmaceutically effective amount of a second generation
34 monoclonal antibody of the present invention, an

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1 immunoreactive fragment or recombinant thereof conjugated
2 to a therapeutic agent.

3 BRIEF DESCRIPTION OF THE DRAWINGS

4 Figure 1 is a schematic diagram of: (1) the
5 differential binding specificities of the CC and MATAG
6 monoclonal antibodies of the present invention to LS-174T
7 colon carcinoma cells (ATCC No. CRL-188) in a competition
8 radioimmunoassay (hereinafter "RIA") with B72.3; (2) the
9 isotypes of the CC and MATAG monoclonal antibodies of the
10 present invention; and (3) the binding specificity of the
11 CC and MATAG monoclonal antibodies of the present
12 invention to various colon carcinomas in a solid phase
13 RIA (hereinafter "SPRIA").

14 Figure 2 is an analysis of the binding specificity
15 of monoclonal antibody CC41 to LS-174T colon carcinoma
16 cell extract in a competition RIA with B72.3. Figure 2B
17 is a quantitative analysis of the binding specificities
18 of monoclonal antibodies B72.3 and CC41 to LS-174T colon
19 carcinoma cell line extract (LS) and a breast carcinoma
20 biopsy extract (Br. Ca.) in a SPIRA.

21 Figure 2C is an analysis of the binding
22 specificity of monoclonal antibody CC60 to LS-174T colon
23 carcinoma cell extract in a competition RIA with B72.3.
24 Figure 2D is a quantitative analysis of the binding
25 specificities of monoclonal antibodies B72.3 and CC60 to
26 LS-174T colon carcinoma cell line extract (LS) and a
27 breast biopsy extract (Br. Ca.) in a SPIRA.

28 Figure 2E is an analysis of the binding
29 specificity of monoclonal antibody CC83 to LS-174T colon
30 carcinoma cell extract in a competition RIA with B72.3.
31 Figure 2F is a quantitative analysis of the binding
32 specificities of monoclonal antibodies B72.3 and CC83 to

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1 LS-174T colon carcinoma cell line extract (LS) and a
2 breast carcinoma biopsy extract (Br. Ca.) in a SPRIA.

3 Figure 2G is an analysis of the binding
4 specificity of monoclonal antibody CC49 to LS-174T colon
5 carcinoma cell extract in a competition RIA with B72.3.
6 Figure 2H is a quantitative analysis of the binding
7 specificities of monoclonal antibodies B72.3 and CC49 to
8 LS-174T colon carcinoma cell line extract (LS) and a
9 breast carcinoma biopsy extract (Br. Ca.) in a SPRIA.

10 Figure 3 is an analysis of a competition RIA with
11 CC49, wherein ^{125}I -labelled CC49 monoclonal antibody was
12 reacted with LS-174T colon carcinoma cell extract and
13 purified CC30, CC46, CC49, CC83 and B72.3 were used as
14 competing antibodies.

15 Figure 4A is an analysis of the in vivo targeting
16 of a LS-174T colon carcinoma xenograft with monoclonal
17 antibody CC11.

18 Figure 4B is an analysis of the in vivo targeting
19 of a LS-174T colon carcinoma xenograft with monoclonal
20 antibody CC46.

21 DETAILED DESCRIPTION OF THE INVENTION

22 I. Characteristics of the Monoclonal Antibodies

23 The monoclonal antibodies specifically developed
24 in the present invention, designated CC1 to CC92 (IgG
25 monoclonal antibodies) and MATAG 1 to MATAG 18 (IgM
26 monoclonal antibodies) (see Figure 1) all have binding
27 specificity to TAG-72 and numerous types of human
28 carcinomas (including breast, ovarian, lung, colorectal,
29 endometrial and pancreatic carcinomas), and are different
30 from B72.3 in that they:

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1 (1) have binding specificity to more human
2 carcinomas than B72.3 while still maintaining essentially
3 no specificity to normal adult human tissues;

4 (2) have a higher binding affinity for
5 TAG-72 than B72.3, i.e., on the order of greater than 3×10^9 M,
6 preferably greater than 8×10^9 M and consequently
7 bind human carcinomas in vivo at a higher efficiency;

8 (3) exhibit a 50% or more efficiency than
9 B72.3 in targeting human carcinomas in situ (i.e., 50%
10 more injected dose/gram tumor than B72.3 and preferably
11 greater than 100% more injected dose/gram tumor than
12 B72.3);

13 (4) can be easily fragmented with pepsin to
14 obtain $F(ab')_2$, $F(ab')$ and $F(ab)$ fragments that are
15 highly immunoreactive; and

16 (5) include monoclonal antibodies of the
17 IgG_{2a}, IgG_{2b}, and IgM isotypes so they can more
18 efficiently be used in monoclonal antibody targeted
19 effector cell mediated cytotoxicity or complement
20 mediated cytotoxicity studies.

21 The development of the CC and MATAG monoclonal
22 antibodies of the present invention also now makes
23 feasible the use of double determinant RIAs (hereinafter
24 "DDRIA"s) for more efficient detection of human carcinoma
25 antigens in body fluids and biopsies of cancer patients.

26 II. Production of the Monoclonal Antibodies

27 The CC and MATAG monoclonal antibodies of the
28 present invention are produced by immunizing mice (or
29 other animals such as rats, rabbits, goats, and humans)
30 with purified TAG-72 obtained from various xenografts,
31 such as LS-174T human colon carcinoma xenografts prepared

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1 using LS-174T carcinoma cells (ATCC No. CRL-188) and
2 OVCAR-3 human ovarian cancer xenografts, prepared using
3 OVCAR-3 carcinoma cells (see Hamilton, et al., Cancer
4 Res., 43:5379-5389 (1983)).

5 TAG-72 is purified from the xenografts by methods
6 well known in the art. More specifically, by the
7 following steps: (1) breaking the cells; (2)
8 centrifuging and/or filtering to remove cellular debris;
9 (3) carrying out sizing column chromatography to obtain
10 proteins having a molecular weight of $>10^6$ d, i.e., the
11 molecular weight of TAG-72; and then (4) carrying out
12 B72.3 affinity column chromatography to obtain the
13 desired TAG-72 (see Paterson, et al., Intl. J. Cancer,
14 37:659-666 (1986)).

15 Immunizing the animals, e.g., mice, with the
16 purified TAG-72, isolating the immunized cells, fusing
17 the immunized cells with mouse myeloma cells (or myeloma
18 cells of other species such as rats, rabbits, goats and
19 humans), all of which are well known in the art and
20 readily available, and culturing the resulting fused
21 cells under conditions which allow for growth of
22 hybridomas, are all conducted by methods well known or
23 readily determined in the art (see Herzenberg, et al.,
24 "Handbook of Experimental Immunology", Oxford, Blackwell,
25 pp. 25.1-25.7; Colcher, et al., Proc. Natl. Acad. Sci.
26 USA, 78:3199-3203 (1981); and Muraro, et al., Intl. J.
27 Cancer, 39:34-44 (1987)).

28 The resulting hybridomas are then tested to
29 isolate those which produce monoclonal antibodies having
30 binding specificity to TAG-72 and human carcinomas but
31 not to normal adult human tissues. This screening is
32 carried out using a SPRIA as described in greater detail
33 in the Examples provided hereinafter.

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1 The binding affinity of monoclonal antibodies for
2 TAG-72 is determined by means well known in the art (see
3 Heyman, et al., J. Immunol. Methods, 68:193-204 (1984))
4 and as described in detail in the Examples provided
5 hereinafter.

6 The isotypes (IgG₁, IgG_{2a}, IgG_{2b}, IgG₃ or IgM) of
7 the monoclonal antibodies are determined by means well
8 known in the art (see Colcher, et al., Cancer Res.,
9 41:1451-1459 (1981)) and as described in detail in the
10 Examples provided hereinafter.

11 In the non-limiting Examples provided hereinafter,
12 in excess of four thousand hybridomas were produced by
13 fusing (i) spleen cells of mice immunized with purified
14 TAG-72 which was obtained from a LS-174T human colon
15 carcinoma xenograft, and (ii) the well known and readily
16 available NS-1 mouse myeloma line (ATCC No. TIB-18).
17 From these hybridomas, 44 double cloned hybridomas (29 CC
18 second generation monoclonal antibodies and 15 MATAG
19 second generation monoclonal antibodies) were selected
20 and characterized as described in the Examples provided
21 hereinafter.

22 The CC monoclonal antibodies of the present
23 invention are fragmented to obtain highly immunoreactive
24 F(ab')₂ and F(ab) fragments using the enzyme pepsin by
25 methods well known in the art (see Colcher, et al.,
26 Cancer Res., 43:736-742 (1983)) and as described in
27 greater detail in the Examples provided hereinafter.
28 The immunoreactivity of the resulting F(ab')₂, F(ab') and
29 F(ab) fragments are determined in a competition RIA or
30 SPRIA as described above for the complete monoclonal
31 antibody molecule.

32 The second generation antibodies of the present
33 invention are also made into recombinant forms by

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1 techniques of molecular biology well known in the art
2 (see Rice, et al., Proc. Natl. Acad. Sci. USA, 79:7862-
3 7865 (1982); Kurokawa, et al., Nucleic Acids Res., 11:
4 3077-3085 (1983); Oi, et al., Proc. Natl. Acad. Sci. USA,
5 80:825-829 (1983); Boxx, et al. Nucleic Acids Res., 12:
6 3791-3806 (1984); Boulianne, et al., Nature (London),
7 312:643-646 (1984); Cabily, et al., Proc. Natl. Acad.
8 Sci. USA, 81:3273-3277 (1984); Kenten, et al. Proc. Natl.
9 Acad. Sci. USA, 81:2955-2959 (1984); Liu, et al., Proc.
10 Natl. Acad. Sci. USA, 81: 5369-5373 (1984); Morrison, et
11 al., Proc. Natl. Acad. Sci. USA, 81:6851-6855 (1984);
12 Neuberger, et al., Nature (London), 312:604-608 (1984);
13 Potter, et al., Proc. Natl. Acad. Sci. USA, 81:7161-7165
14 (1984); Neuberger, et al., Nature (London), 314:268-270
15 (1985); Jones, et al., Nature (London), 321:522-525
16 (1986); Oi, et al., BioTechniques, 4:214-221 (1986);
17 Sahagan, et al., J. Immunol., 137:1066-1074 (1986); Sun,
18 et al., Hybridoma 5 (Suppl. 1):S-17-S20 (1986); and Sun,
19 et al., Proc. Natl. Acad. Sci. USA, 84:214-218 (1987));
20 all of which are specifically incorporated herein by
21 reference.

22 More specifically, the second generation
23 monoclonal antibodies of the present invention are
24 altered to a chimeric form by substituting, e.g., human
25 constant regions (F_C domains) for mouse constant regions
26 by recombinant DNA techniques known in the art as
27 described in the above cited references. These F_C
28 domains can be of various human isotypes, i.e., IgG₁,
29 IgG₂, IgG₃, IgG₄, or IgM.

30 In addition, the second generation monoclonal
31 antibodies of the present invention are altered to an
32 affinity modified form, avidity modified form, or both,
33 by altering binding sites or altering the hinge region

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1 using recombinant DNA techniques well known in the art as
2 described in the above cited references.

3 The recombinant antibody forms are also fragmented
4 to produce immunoreactive fragments $F(ab')_2$, $F(ab')$, or
5 $F(ab)$ in the same manner as described above in which the
6 second generation monoclonal antibodies of the present
7 invention are fragmented.

8 Accordingly, as used herein, the expression
9 "recombinant antibodies" collectively includes chimeric/
10 recombinant forms of the second generation monoclonal
11 antibody of the present invention wherein the F_C domain
12 is substituted for an F_C domain of another species or
13 isotype, affinity modified forms of the second generation
14 monoclonal antibody of the present invention wherein the
15 binding sites are altered, avidity modified forms of the
16 second generation monoclonal antibody of the present
17 invention wherein the hinge regions are altered,
18 immunoreactive fragments thereof and combinations
19 thereof.

20 The second generation monoclonal antibodies of the
21 present invention are produced in large quantities by
22 injecting a hybridoma producing a second generation
23 monoclonal antibody of the present invention into the
24 peritoneal cavity of pristane-primed mice, and after an
25 appropriate time (about 1-2 weeks), harvesting ascites
26 fluid from the mice, which yields a very high titer of
27 homogenous monoclonal antibody, and isolating the
28 monoclonal antibodies therefrom by methods well known in
29 the art (see Stramignoni, et al., Intl. J. Cancer, 31:
30 543-552 (1983)). Alternatively, the second generation
31 monoclonal antibodies are produced by culturing a
32 hybridoma producing a second generation monoclonal
33 antibody of the present invention in vitro and isolating
34 secreted monoclonal antibodies from the cell culture

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1 medium by methods well known in the art (see Colcher, et
2 al., Proc. Natl. Acad. Sci. USA, 78:3199-3203 (1981)).

3 The CC and MATAG monoclonal antibodies of the
4 present invention are thus produced according to the
5 above method. The binding specificity and binding
6 affinity of these monoclonal antibodies and a comparison
7 of such with B72.3 are discussed in greater detail in the
8 Examples provided hereinafter.

9 III. Uses of the Monoclonal Antibodies

10 The second generation monoclonal antibodies of the
11 present invention, immunoreactive fragments or
12 recombinants thereof, can be used either alone, in
13 combination with one another, or in combination with
14 other antibodies, such as B72.3 or immunoreactive
15 fragments thereof, in: (1) in vitro diagnostic assays
16 using labelled monoclonal antibodies for the detection of
17 TAG-72 in body fluids of patients; (2) in vivo diagnostic
18 assays (diagnostic imaging) using the second generation
19 monoclonal antibodies of the present invention,
20 immunoreactive fragments or recombinants thereof,
21 conjugated to an imaging marker, for the in situ
22 detection of carcinoma lesions; (3) in vivo cancer
23 treatment using the second generation monoclonal
24 antibodies of the present invention, immunoreactive
25 fragments or recombinants thereof alone or conjugated to
26 a therapeutic agent such as radionuclide, drug, toxin,
27 effector cells, other antibodies or via a complement
28 mechanism; (4) immunohistopathology or
29 immunocytochemistry for the detection or phenotyping of
30 carcinoma cells; and (5) as immunogens to activate the
31 anti-idiotypic network for active immunotherapy against
32 carcinomas.

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1 A. In Vitro Diagnostic Assays

2 In vitro diagnostic assays of human carcinomas or
3 metastases thereof by detecting TAG-72 in body fluids of
4 patients using the second generation monoclonal
5 antibodies of the present invention, immunoreactive
6 fragments or recombinants thereof are described in
7 greater detail below.

8 The body fluid obtained from a patient is
9 contacted with the monoclonal antibody of the present
10 invention, immunoreactive fragment or recombinant
11 thereof. A diagnosis is then made by determining the
12 amount of monoclonal antibody, immunoreactive fragment or
13 recombinant thereof binding to substances (TAG-72)
14 present in the body fluid and comparing the amount of
15 monoclonal antibody, immunoreactive fragments or
16 recombinants thereof bound to the body fluid substances
17 to a predetermined base level as hereinafter defined.
18 The amount of bound monoclonal antibody, immunoreactive
19 fragment or recombinant thereof exceeding the base level
20 indicates the presence of a human carcinoma or metastases
21 thereof.

22 Examples of body fluids which can be used in the
23 in vitro method are any body fluids suspected of
24 containing TAG-72. Preferred examples thereof include
25 blood (serum or plasma), sputum, nipple discharge, cyst
26 fluid, ascites fluids, pleural effusions, seminal plasma,
27 semen, urine and prostatic fluid and/or biopsy specimens.
28 Serum or plasma are the more preferred body fluids
29 employed in the present invention. The body fluids can
30 be obtained by methods readily known to or determined by
31 those skilled in the art.

32 The body fluid is contacted with the second
33 generation monoclonal antibody of the present invention,

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1 immunoreactive fragment or recombinant thereof and the
2 amount of monoclonal antibody, immunoreactive fragment or
3 recombinant thereof bound to substances in the body fluid
4 is determined by means of immunochemical assays well
5 known to those skilled in the art, as described, for
6 example, in Klug, et al., Cancer Res., 44:1048-1053
7 (1984); Klug, et al., Intl. J. Cancer, 38:661-669 (1986);
8 Herlyn, et al., J. Clin. Immunol., 2:135-140 (1982);
9 Metzgar, et al., Proc. Natl. Acad. Sci. USA, 81:5242-5246
10 (1984); Papsidero, et al., Cancer Res., 44:4653-4657
11 (1984); Hayes, et al., J. Clin. Invest., 75:1671-1678
12 (1985); Killian, et al., Cancer Res., 45:886-891 (1985);
13 Hedin, et al., Proc. Natl. Acad. Sci. USA, 80:3470-3474
14 (1983); Pekary, et al., Clin. Chem., 30:1213-1215 (1984);
15 Bast, et al., New England J. Med., 309:883-887 (1983);
16 and Bellet, et al., Proc. Natl. Acad. Sci. USA, 81:
17 3869-3873 (1984), the disclosures of all of which are
18 specifically incorporated herein by reference.

19 An example of one type of immunochemical assay
20 useful in the present invention is a sandwich
21 immunoradiometric assay (hereinafter "IRMA"). In this
22 type of assay, the presence of antigen (TAG-72) is
23 measured directly by reacting it with an excess of
24 labelled monoclonal antibody. In such an assay, before
25 the antigen is reacted with the labelled monoclonal
26 antibody, the antigen is insolubilized on an
27 immunoabsorbent which specifically binds the antigen.
28 The immunoabsorbent is formed by affixing a second
29 generation monoclonal antibody, immunoreactive fragment
30 or recombinant thereof to a substrate such as an
31 immunobead. In sandwich assays for an antigen which is
32 monomeric, two antibodies which recognize distinct
33 epitopes on the antigen are required, i.e., a so-called
34 "double determinant" assay, so that there is no

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1 competition for binding to the antigen. In sandwich
2 assays, one antibody is bound to the immunoabsorbent and
3 the other antibody is used as the labelled tracer. In
4 assays for dimeric or polymeric antigens, the same
5 antibody can be bound to the immunoabsorbent as the
6 labelled tracer.

7 Sandwich IRMA's may be performed in a forward,
8 reverse or simultaneous mode.

9 In a forward sandwich assay for TAG-72, a
10 monoclonal antibody is affixed to a solid phase such as
11 an immunobead to form an immunoabsorbent specific for
12 TAG-72. A body liquid sample containing TAG-72 is then
13 incubated with the immunoabsorbent. Incubation is
14 maintained for a sufficient period of time to allow
15 TAG-72 in the body fluid to bind to the immobilized
16 monoclonal antibody on the immunoabsorbent. After this
17 first incubation, the solid phase immunoabsorbent is
18 separated from the incubation mixture. The
19 immunoabsorbent may be washed to remove unbound
20 interfering substances, such as non-specific binding
21 proteins, which may also be present in the body fluid.
22 The immunoabsorbent containing TAG-72 bound to an
23 immobilized monoclonal antibody is subsequently incubated
24 with a labelled monoclonal antibody, immunoreactive
25 fragment or recombinant thereof. Again, the incubation
26 is carried out for a period of time and under conditions
27 sufficient to ensure binding of the labelled monoclonal
28 antibody, immunoreactive fragment or recombinant thereof
29 to TAG-72. After the second incubation, another wash may
30 be performed to remove unbound labelled monoclonal
31 antibody, immunoreactive fragment or recombinant thereof
32 from the solid phase immunoabsorbent. The labelled
33 monoclonal antibody, immunoreactive fragment or
34 recombinant thereof bound to the solid phase

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1 immunoadsorbent is then measured, and the amount of
2 labelled monoclonal antibody, immunoreactive fragment or
3 recombinant thereof detected serves as a direct measure
4 of the amount of TAG-72 present in the body fluid.

5 The sandwich IRMA may also be performed in reverse
6 and simultaneous modes. In the reverse mode, an
7 incubation mixture is formed of the body fluid to be
8 tested and soluble labelled monoclonal antibody,
9 immunoreactive fragment or recombinant thereof directed
10 against TAG-72. The mixture is incubated, then
11 contacted with a solid phase immunoadsorbent also
12 containing a monoclonal antibody, immunoreactive fragment
13 or recombinant thereof directed against TAG-72. After
14 another incubation, the immunoadsorbent is separated from
15 the mixture and the label bound to the immunadsorbent is
16 taken as an indication of the amount of TAG-72 in the
17 body fluid.

18 In the simultaneous mode, an incubation mixture is
19 formed of the body fluid, the labelled monoclonal
20 antibody, immunoreactive fragment or recombinant thereof
21 and the solid phase immunoadsorbent. After incubation
22 for a sufficient time, the solid phase immunoadsorbent is
23 separated from the mixture and the label associated with
24 the immunoadsorbent is measured to give an indication of
25 the amount of TAG-72 in the body fluid.

26 For each incubation step in the various assay
27 modes described above, the time and conditions of
28 incubation are selected to ensure maximum binding of
29 TAG-72 to the immobilized monoclonal antibody,
30 immunoreactive fragment or recombinant thereof and to
31 labelled monoclonal antibody, immunoreactive fragment or
32 recombinant thereof, but generally are about 6 to 16
33 hours at room temperature (22° to 27°C).

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1 In addition to the IRMA's described above, other
2 immunoassays useful in the present invention include
3 competitive binding assays such as RIAs and fluorescent
4 or enzymelinked immunoassays (hereinafter "ELISA"). On
5 suitable type of RIA is a SPRIA.

6 For a SPRIA, a solid phase immunoabsorbent is
7 prepared as described for the IRMA.

8 The immunoabsorbent is then incubated with the
9 body fluid and a known amount of labelled TAG-72 for a
10 period of time and under conditions which permit binding
11 of TAG-72 to the immunoabsorbent. The immunoabsorbent is
12 separated from the body fluid and the amount of label
13 associated therewith is assessed. By reference to a pre-
14 established inhibition curve defining the relationship
15 between labelled TAG-72 associated with the
16 immunoabsorbent, the amount of unlabelled human TAG-72 in
17 the body fluid is determined.

18 In the various SPRIA's, the immunoabsorbent is
19 separated from incubation mixtures containing the body
20 fluid, the labelled antibody or both. Separation can be
21 accomplished by any conventional separation technique
22 such as sedimentation or centrifugation. Preferably,
23 though not necessarily, the immunoabsorbent is washed
24 prior to contacting it, when required, with a second
25 incubation medium and prior to measuring the amount of
26 label associated with the immunoabsorbent. The washing
27 removes non-specific interfering substances or excess
28 labelled antibody which may affect the accuracy and
29 sensitivity of the assay.

30 The particular label employed to label the second
31 generation monoclonal antibodies of the present
32 invention, immunoreactive fragments or recombinants
33 thereof or TAG-72 in the above-described assays is not
34 critical to the present invention and can be a

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1 radioisotope such as ^{32}P , ^{14}C , ^3H , ^{125}I , ^{131}I , or ^{35}S for
2 the IRMA and RIA or a fluorescent molecule such as
3 fluorescein or rhodamine or an enzyme, which, under the
4 presence of an appropriate substrate converts the
5 substrate to a color product for the ELISA. Examples of
6 such enzymes include alkaline phosphatase and horseradish
7 peroxidase.

8 As the last step in the in vitro diagnostic method
9 according the present invention, the amount of second
10 generation monoclonal antibody, immunoreactive fragment
11 or recombinant thereof, binding to substances (TAG-72)
12 present in the body fluid is compared to a predetermined
13 base level.

14 The determination of the base level of monoclonal
15 antibody assay binding to be expected is a determination
16 routinely made by those of ordinary skill in the art when
17 defining the parameters necessary for the reading of a
18 diagnostic test of this sort. These determinations may
19 be made without undue experimentation, particularly in
20 light of the teachings set forth herein.

21 Generally, the "base level" is defined as (1) two
22 standard deviations above the mean of the normal
23 population, or (2) the level below which 99% of the
24 normal population falls.

25 B. In Vivo Diagnostic Assays

26 In vivo diagnostic assay of human carcinomas or
27 metastases thereof using the second generation monoclonal
28 antibodies of the present invention, immunoreactive
29 fragments or recombinants thereof, are described in more
30 detail below.

31 A second generation monoclonal antibody of the
32 present invention, immunoreactive fragment or recombinant

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1 thereof, conjugated to an imaging marker is administered
2 to a patient (or subsequently administering the marker or
3 linker conjugate marker after administration of the
4 second generation monoclonal antibody) and then the
5 presence of the imaging marker in the patient is detected
6 by exposing the patient to an appropriate means for
7 detecting the marker.

8 Administration and detection of the antibody-
9 imaging marker conjugate as well as a methods of
10 conjugation of the antibody to the imaging marker are
11 accomplished by methods readily known to or readily
12 determined by those skilled in the art, as described, for
13 example, in Goldenberg, et al., New England J. Med., 298:
14 1384-1388 (1978); Goldenberg, et al., J.A.M.A., 250:630-
15 635 (1983); Goldenberg, et al., Gastroenterol., 84:524-
16 532 (1983); Siccardi, et al., Cancer Res., 45:4817-4822
17 (1986); Epenetos, et al., Cancer, 55:984-987 (1985);
18 Philben, et al., Cancer, 57:571-576 (1986); Chiou, et
19 al., Cancer Res., 45:6140-6146 (1985); Hwang, et al., J.
20 Natl. Cancer Inst., 76:849-855 (1986); Colcher, et al.,
21 Cancer Res., 43:736-742 (1983); Colcher, et al.,
22 "Laboratory Research Methods in Biology and Medicine
23 Immunodiagnosics", New York, Alan R. Liss, pp. 215-258
24 (1983); Keenan, et al., J. Nucl. Med., 25:1197-1203
25 (1984); Colcher, et al., Cancer Res., 43:1185-1189
26 (1987); Esteban, et al., Intl. J. Cancer, 39:50-59
27 (1987); Martin, et al., Curr. Surg., 41:193-194 (1984);
28 Martin, et al., Hybridoma, 5:S97-S108 (1986); and Martin,
29 et al., Am. J. Surg., 150:672-675 (1985); the disclosures
30 of all of which are specifically incorporated herein by
31 reference.

32 The dosage will vary depending upon the age and
33 weight of the patient, but generally a one time dosage of
34 about 0.1 to 20 mg of antibody-marker conjugate per

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1 patient is sufficient. A more preferred dosage is about
2 1.0 to 2.0 mg of antibody-marker conjugate per patient.

3 Examples of imaging markers which can be
4 conjugated to the antibody are well known to those
5 skilled in the art and include substances which can be
6 detected by diagnostic imaging using a gamma scanner or
7 hand held gamma probe or Positron Emission Tomography or
8 the like as described by the references cited above and
9 substances which can be detected by nuclear magnetic
10 resonance imaging using a nuclear magnetic resonance
11 spectrometer or the like as described in the references
12 cited above.

13 Suitable examples of substances which can be
14 detected using a gamma scanner or the like include ^{125}I ,
15 ^{131}I , ^{123}I , ^{111}In , and $^{99\text{m}}\text{Tc}$. ^{111}Tl and $^{99\text{m}}\text{Tc}$ are
16 preferred due to their low energy and suitability for
17 long range detection.

18 An example of a substance which can be detected
19 using a nuclear magnetic resonance spectrometer or the
20 like is the nuclear magnetic spin-resonance isotope
21 gadolinium (Gd).

22 C. In Vivo Treatment

23 In vivo treatment of human carcinomas or
24 metastases thereof using second generation monoclonal
25 antibodies of the present invention, immunoreactive
26 fragments or recombinants thereof is described in greater
27 detail below.

28 A pharmaceutically effective amount of a second
29 generation monoclonal antibody of the present invention,
30 immunoreactive fragment or recombinant thereof
31 unconjugated or conjugated to a therapeutic agent is
32 administered to a patient.

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1 Methods of preparing and administering the
2 monoclonal antibody-therapeutic agent conjugate as well
3 as suitable dosages will depend on the age and weight of
4 the patient and the therapeutic agent employed and are
5 well known to or readily determined by those skilled in
6 the art. Representative protocols are described in the
7 references cited below.

8 Examples of the monoclonal antibody-therapeutic
9 agent conjugates which can be used in therapy include
10 antibodies coupled to radionuclides, such as ^{131}T , ^{90}Y ,
11 ^{105}Rh , ^{47}Sc , ^{67}Cu , ^{212}Bi , and ^{211}At , as described, for
12 example in Goldenberg, et al., Cancer Res., 41:4354-4360
13 (1981); Carrasquillo, et al., Cancer Treat. Rep.,
14 68:317-328 (1984); Zalberg, et al., J. Natl. Cancer
15 Inst., 72:697-704 (1984); Jones, et al., Int. J. Cancer,
16 35:715-720 (1985); Lange, et al., Surgery, 98:143-150
17 (1985); Kaltovich, et al., J. Nucl. Med., 27:897 (1986);
18 Order, et al., Intl. J. Radiother. Oncol. Biol. Phys.,
19 8:259-261 (1982); Courtenay-Luck, et al., Lancet,
20 1:1441-1443 (1983); and Ettinger, et al., Cancer Treat.
21 Rep., 66:289-297 (1982), the disclosure of all of which
22 are specifically incorporated herein by reference;
23 antibodies coupled to other drugs or biological response
24 modifiers such as methotrexate, adriamycin, and
25 interferon as described, for example in Chabner, et al.,
26 "Cancer, Principles and Practice of Oncology",
27 Philadelphia, PA, J.B. Lippincott Co., Vol. 1, pp.
28 290-328 (1985); Oldham, et al., "Cancer, Principles and
29 Practice of Oncology", Philadelphia, PA, J.B. Lippincott
30 Co., Vol. 2, pp. 2223-2245 (1985); Deguchi, et al.,
31 Cancer Res., 46:3751-3755 (1986); Deguchi, et al. Fed.
32 Proc., 44:1684 (1985); Embleton, et al., Br. J. Cancer,
33 49:559-565 (1984); and Pimm, et al., Cancer Immunol.
34 Immunother., 12:125-134 (1982), the disclosure

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1 of all of which are specifically incorporated herein by
2 reference; antibodies coupled to toxins, as described,
3 for example, in Uhr, et al., "Monoclonal Antibodies and
4 Cancer", Academic Press, Inc., pp. 85-98 (1983); Vitetta,
5 et al., "Biotechnology and Bio. Frontiers", Ed. P.H.
6 Abelson, pp. 73-85 (1984); and Vitetta, et al., Sci.,
7 219:644-6540 (1983), the disclosures of all of which are
8 specifically incorporated herein by reference;
9 heterobifunctional antibodies for example, antibodies
10 coupled or combined with another antibody so that the
11 complex binds both to the carcinoma and effector cells,
12 e.g., killer cells, such as T cells, as described, for
13 example, in Perez, et al., J. Exper. Med., 163:166-178
14 (1986); and Lau, et al., Proc. Natl. Acad. Sci. USA,
15 82:8648-8652 (1985); the disclosures of both of which are
16 specifically incorporated herein by reference; and
17 native, i.e., non-conjugated or non-complexed, antibody,
18 as described in, for example, Herlyn, et al., Proc. Natl.
19 Acad. Sci. USA, 79:4761-4765 (1982); Schulz, et al.,
20 Proc. Natl. Acad. Sci. USA, 80:5407-5411 (1983); Capone,
21 et al., Proc. Natl. Acad. Sci. USA, 80:7328-7332 (1983);
22 Sears, et al., Cancer Res., 45:5910-5913 (1985); Nepom,
23 et al., Proc. Natl. Acad. Sci. USA, 81:2864-2867 (1984);
24 Koprowski, et al., Proc. Natl. Acad. Sci. USA, 81:216-219
25 (1984); and Houghton, et al., Proc. Natl. Acad. Sci. USA,
26 82:1242-1246 (1985), all of which are specifically
27 incorporated herein by reference.

28 In this method, the monoclonal antibody-
29 therapeutic agent conjugate can be delivered to the
30 carcinoma site thereby directly exposing the carcinoma
31 tissue to the therapeutic agent.

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1 D. Immunohistochemistry and Immunocytochemistry
2 Assays

3 Immunohistochemistry (hereinafter "IHC") and
4 immunocytochemistry (hereinafter "ICC") assays for the
5 diagnosis of human carcinomas or metastases thereof or to
6 make differential diagnoses using the second generation
7 monoclonal antibodies of the present invention, are
8 carried out as described in detail below.

9 A second generation monoclonal antibody of the
10 present invention, is added to a slide containing a 5 μ
11 section of a biopsy specimen (for IHC) or cells (for ICC)
12 from body fluid (such as pleural effusion, ascites,
13 sputum, or vaginal fluid). A series of linkers (e.g.,
14 biotinylated horse anti-mouse IgG followed by avidin
15 DH:biotinylated horseradish peroxidase complex) and dyes
16 (e.g., diaminobenzidine) are then added to the slides to
17 detect binding of the second generation monoclonal
18 antibody, immunoreactive fragment or recombinant thereof
19 to carcinoma cells in the biopsy or body fluid by a color
20 reaction, i.e., carcinoma cells will look reddish-brown
21 while normal and benign cells will look blue (the
22 background stain). Alternate linkers, dyes and
23 subsequent color reactions, may of course be applied, as
24 incorporated by reference herein (see Sternberger,
25 "Immunocytochemistry", New York, John Wiley & Sons,
26 Second Edition, pp. 82-169 (1979)). By this method:
27 (a) carcinoma cells can be detected in biopsy specimens
28 and body fluids as an adjunct to making a diagnosis of
29 cancer, and (b) a differential diagnosis can be made; for
30 example, TAG-72 has been shown to be present in
31 adenocarcinoma of the lung and adenosquamous carcinoma of
32 the lung but not in small cell carcinoma. Thus,
33 detection of binding of the second generation monoclonal

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1 antibody of the present invention, immunoreactive
2 fragment or recombinant thereof to a lung biopsy would
3 rule out small cell lung cancer. Furthermore, since
4 TAG-72 has been shown not to be expressed in malignant
5 mesothelioma, the second generation monoclonal antibody
6 of the present invention, therefore can be used to
7 differentiate adenocarcinoma of the lung from malignant
8 mesothelioma.

9 The use of IHC and ICC assays for the diagnosis of
10 cancer or to make differential diagnoses are accomplished
11 by methods known or readily determined by those skilled
12 in the art, as described, for example, in Nuti, et al.,
13 Intl. J. Cancer, 29:539-545 (1982); Stramignoni, et al.,
14 Intl. J. Cancer, 31:543-552 (1983); Szpak, et al., Acta
15 Cytologica, 28:356-367 (1984); Johnston, et al., Cancer
16 Res., 45:1894-1900 (1985); Szpak, et al., Am. J. Path.,
17 122:252-260 (1986); Martin, et al., Am. J. Clin. Path.,
18 86:10-18 (1986); Nuti, et al., Intl. J. Cancer, 37:493-
19 498 (1986); Johnston, et al., Cancer Res., 46:850-857
20 (1986); Thor, et al., Cancer Res., 46:3118-3124 (1986);
21 Ohuchi, et al., Intl. J. Cancer, 38:643-650 (1986);
22 Johnston, et al., Cancer Res., 45:6462-6470 (1986); and
23 Thor, et al., Cancer Res., 47:505-512 (1987), the
24 disclosures of all of which are specifically incorporated
25 herein by reference.

26 The amount of second generation monoclonal
27 antibody of the present invention, used per slide and the
28 incubation time and temperature may vary, but generally,
29 the IHC and ICC assays are conducted at about 4°C for
30 about 18 hours using about 40 µg per ml of monoclonal
31 antibody.

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1 E. Activating the Anti-Idiotypic Network

2 Activating the anti-idiotypic network for cancer
3 therapy using the second generation monoclonal antibodies
4 of the present invention, immunoreactive fragments or
5 recombinants thereof is carried out as described in
6 detail below.

7 A second generation monoclonal antibody of the
8 present invention, immunoreactive fragment or recombinant
9 thereof (designated Ab 1) is administered to a patient at
10 multiple intervals. The immune system of the patient
11 will respond by the generation of antibodies (designated
12 Ab 2) which having binding specificity to the binding
13 site of Ab 1. These anti-idiotypic antibodies (Ab 2's)
14 will then elicit the formation of antibodies (designated
15 Ab 3) which have binding specificity to the binding site
16 of Ab 2. The Ab 2 antibodies will be an internal image
17 of the original TAG-72, and thus the Ab 3 antibodies will
18 have binding specificity and potentially destroy a
19 carcinoma producing TAG-72.

20 The use of monoclonal antibodies to activate the
21 idiotypic network and the procedures used to accomplish
22 this are readily known or readily determined by those
23 skilled in the art, as described, for example, in
24 Nisonoff, et al., Clin. Immunol. and Path., 21:397-406
25 (1981), Forstrom, et al., Nature, 303:627-629 (1983);
26 Kauffman, et al., J. Immunol., 131:2539-2541 (1983);
27 Reagen, et al., J. Virol., 48:660-666 (1983); Koprowski,
28 et al., Proc. Natl. Acad. Sci. USA, 81:216-219 (1984),
29 Herlyn, et al., J. Immunol., 143:1300-1304 (1985);
30 Koprowski, et al., J. Immunol. Metho., 85:27-38 (1985),
31 Koprowski, et al., Science, 232:100-102 (1985); Greene,
32 et al., J. Immunol., 137:2930-2936 (1986), Kohler, et
33 al., J. Immunol., 137:1743-1749 (1986), Notkins, et al.,

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1 J. Exp. Med., 163:1355-1360 (1986), the disclosures of
2 all of which are specifically incorporated herein by
3 reference.

4 The activation of the anti-idiotypic network can
5 be used to stimulate a patient's immune system so that
6 the patient can mount an active immune response against
7 carcinomas producing TAG-72.

8 The following examples are provided for
9 illustrative purposes only and are in no way intended to
10 limit the scope of the present invention.

11 Example 1

12 Preparation of Monoclonal Antibodies

13 A. Preparation of Immunogen

14 LS-174T colon carcinoma cells (ATCC No. CRL-188)
15 were grown in Eagle's minimum essential medium with
16 non-essential amino acids supplemented with 10% (v/v)
17 heat-inactivated fetal calf serum, 100 units/ml
18 penicillin and 100 µg/ml streptomycin. The LS-174T cells
19 were tested for the presence of Mycoplasma species and
20 were found to be negative.

21 Four-week old female athymic mice were inoculated
22 subcutaneously with 1×10^6 LS-174T cells in 0.1 ml of
23 culture medium. Carcinoma xenografts were harvested when
24 they reached approximately 1.0 cm in diameter (15-20 days
25 after cell implantation), quick frozen in liquid nitrogen
26 and stored at -70°C. Large carcinoma xenografts were not
27 used due to necrosis.

28 Thereafter, approximately 3 grams of frozen
29 LS-174T human carcinoma xenograft was homogenized with an
30 Omni Mixer for 45 sec in buffer comprising 20 mM Tris (pH
31 7.2) and 150 mM NaCl (hereinafter "TBS"). The

- 30 -

1 homogenized xenograft was then filtered through glass
2 wool and loaded onto a Sepharose CL-4B column sizing
3 column (Pharmacia, Upsala, Sweden) (5.5. x 25 cm) which
4 was previously equilibrated in TBS. The column was
5 eluted using TBS (pH 7.2).

6 7.0 ml fractions were collected and examined in a
7 direct binding assay 1/10 volume dilutions. More
8 specifically, 50 μ l of the dilutions were added to wells
9 of a 96-well polyvinyl chloride microtiter plate
10 (Dynatech Laboratories, Inc., Alexandria VA). To
11 minimize nonspecific protein adsorption, the microtiter
12 wells were treated with 100 μ l of 5.0% bovine serum
13 albumin (hereinafter "BSA") in phosphate buffered saline,
14 comprising 8.0 mM Na_2HPO_4 , 2.5 mM KCl, 140 mM NaCl, 0.5
15 mM MgCl_2 , 1.0 mM CaCl_2 , (pH 7.2) (hereinafter "PBS") and
16 incubated for 1 hour at 37°C. Next, the BSA was removed
17 and ^{125}I -B72.3, prepared as described in Colcher, et al.,
18 Cancer Res., 44:5744-5751 (1984) at 50,000 cpm/25 μ l per
19 well, was added to each well. Following an overnight
20 incubation at 4°C, unbound ^{125}I -B72.3 was removed by
21 washing with 1.0% BSA (v/v) in PBS. The bound ^{125}I -B72.3
22 was detected by cutting individual wells from the plate
23 and measuring the radioactivity in a gamma counter
24 (RIAGamma, LKB, Bromma, Sweden).

25 Thereafter, the peak fractions were pooled (130
26 mls of material), and loaded onto a B72.3 affinity column
27 which was washed with TBS. The B72.3 affinity column was
28 prepared as described in Johnson, et al., Cancer Res.,
29 46:850-857 (1986) and comprised 100 ml of 1,1'-carbonyl-
30 diimidazole activated affinity matrix Reacta-Gel HW65F
31 (Pierce, Rockford, IL) coupled with 200 mg of B72.3. The
32 column was washed with TBS and the bound protein was

- 31 -

1 eluted with 3.0 M NaI in TBS. The column was finally
2 washed with TBS.

3 5.0 ml fractions were collected and examined in a
4 second direct binding assay carried out as described
5 above. The peak fractions were pooled (92 mls of
6 protein) dialyzed against 4.0 liters of 20 mM Tris (pH
7 7.2) at 4°C overnight. The purified TAG-72 thus obtained
8 was concentrated in Aquacide II, sodium salt of
9 carboxymethyl cellulose (Calbiochem, San Diego, CA) and
10 used as the immunogen.

11 B. Immunizations

12 1. CC Group

13 For the group designated CC hereinafter,
14 three four-week old BALB/c mice were immunized by
15 intraperitoneal inoculation of 10 µg of TAG-72 purified
16 as described above which had been pre-mixed with an equal
17 volume of complete Freund's adjuvant. After 80 days,
18 the mice received booster doses intraperitoneally of 50
19 µg of TAG-72 purified as described above which had been
20 pre-mixed with an equal volume of incomplete Freund's
21 adjuvant. Seven days later the mice received 10 µg of
22 TAG-72 in saline, by intravenous inoculation. Spleens
23 were harvested three days later for cell infusion.

24 2. MATAG Group

25 For the group designated MATAG hereinafter,
26 two four-week old BALB/c mice were immunized by
27 intraperitoneal inoculation of 50 µg of TAG-72 purified
28 as described above which had been pre-mixed with an equal
29 volume of complete Freund's adjuvant. After seven days,
30 the mice received booster doses intraperitoneally of 50
31 µg of TAG-72 purified as described above which had been
32 pre-mixed with an equal volume of incomplete Freund's

- 32 -

1 adjuvant. Seven days later the mice received 10 µg of
2 TAG-72 in saline, by intravenous inoculation. Spleens
3 were harvested three days later for cell fusion.

4 C. Preparation of Hybridomas

5 Somatic cell hybrids (hybridomas) were prepared
6 using a modification of the method of Herzenberg, et al.,
7 "Handbook of Experimental Immunology", Oxford, Blackwell,
8 pp. 25.1-25.7 (1978). More specifically, single cell
9 suspensions of spleen cells from the immunized mice were
10 made by passing the spleen tissue of the mice through a
11 No. 3 mesh stainless steel screen (B. Fenenco Co., Inc.,
12 Norchester, MA). The spleen cells and NS-1 mouse myeloma
13 cells (ATCC No. TIB-18) were washed in RPMI-1640 medium,
14 containing 2.0 mM glutamine, 1.0 mM sodium pyruvate, 50
15 units/ml penicillin, 50 µg/ml streptomycin and 0.25 µg/ml
16 Fungizone, an antimycotic mixture (Grand Island
17 Biological Company, Grand Island, NY). Then, the spleen
18 cells and NS-1 mouse myeloma cells were mixed at a 4:1
19 ratio, and fused with 50% (v/v) polyethylene glycol (M.W.
20 1500) (BDH Chemical Ltd., Poole, England). After fusion,
21 individual wells of 96-well microtiter plates (Costar,
22 Cambridge, MA) were seeded with 1×10^6 total cells (0.1
23 ml) of the cell suspension. Fused cells were then
24 selected for growth with HAT media.

25 Cloning of hybridoma cell lines was performed by
26 limiting dilution. Specifically, twenty-four wells of a
27 96-well microtiter plate (Costar, Cambridge, MA) were
28 seeded with one of the following concentrations of
29 hybridoma cells: 10 cells/well, 5 cells/well, 1.0
30 cell/well, or 0.5 cell/well. Mouse thymocytes, derived
31 from the thymus glands of four-week old BALB/c mice, were
32 added to each well as feeder cells at a concentration of
33 10^6 cells/well. Wells were seeded at the

- 33 -

1 concentration that eventually resulted in the growth of
2 single cell cultures.

3 A total of 2,567 initial hybridoma cultures were
4 obtained for the CC group and a total of 2,000 initial
5 hybridoma cultures were obtained for the MATAG group.
6 All hybridoma cell lines selected for further screening
7 were cloned twice.

8 D. Solid Phase Radioimmunoassays

9 1. CC Group

10 The CC group was assayed in a SPRIA using
11 the cell extracts from a metastatic breast carcinoma and
12 normal spleen and liver.

13 More specifically, 50 μ l of the cell
14 extracts (5 μ g) were added to each well of a Cooke round
15 bottom polyvinyl chloride microtiter (Dynatech
16 Laboratories, Alexandria, VA) plate and allowed to dry.
17 To minimize non-specific protein adsorption, microtiter
18 wells were treated with 100 μ l of 5.0% (v/v) BSA in PBS
19 and incubated with the sample covered for 1 hour. This
20 and all subsequent incubations were at 37°C. The BSA was
21 then removed and the wells were washed one time with 1.0%
22 (v/v) BSA in PBS. Next, 50 μ l of hybridoma supernatant
23 was added per well. After a 1 hour incubation, the
24 unbound immunoglobulin was removed by washing the plates
25 three times with 1.0% (v/v) BSA in PBS at 100
26 μ l/well/wash.

27 To determine antibody binding, the wells
28 were then incubated with 25 μ l of 125 I-goat-anti-mouse
29 IgG (γ chain specific) (Kirkegaard & Perry, Gaithersburg,
30 MD) at 75,000 cpm/25 μ l per well for 1 hour at 37°C.

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1 The supernatant was aspirated and the plates were washed
2 four times with 1.0% (v/v) BSA in PBS at 100
3 μ l/well/wash.

4 The plates were then subjected to
5 autoradiography using Kodak XAR film and Dupont
6 Lightning-Plus intensifying screens. The films were
7 developed after 16 hours at -70°C. The bound cpm were
8 also detected by cutting the individual wells from the
9 plate and measuring the cpm in a gamma counter.

10 The results yielded 433 CC cultures which
11 had binding specificity in the SPRIA, to the carcinoma
12 extract but not to the normal extracts.

13 All of these 433 CC cultures were then
14 assayed, in a SPRIA as described above, using the cell
15 extracts shown in Table I below.

16 TABLE I

17	Primary colon carcinoma
18	Metastatic breast carcinoma
19	Normal kidney
20	Normal liver
21	Normal colon
22	Normal stomach
23	Normal bone marrow
24	Normal lung
25	Normal thyroid
26	Polymorphonuclear leukocyte
27	Red blood cell

28 The results yielded 99 CC cultures which had
29 binding specificity, in the SPRIA, to the carcinoma
30 extracts but not to the normal extracts listed in Table I
31 above.

32 Next, all of the 99 cultures were cloned
33 into 9,504 wells and each well was checked for growth of
34 a single colony. Those with a single colony were

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1 selected for further assay. Those that were selected
2 were assayed, in a SPRIA as described above, using
3 extracts of a human breast carcinoma and primary colon
4 carcinoma as well as normal liver. The colonies that
5 had binding specificity, in the SPRIA, to the carcinoma
6 extracts but not to normal liver extract were recloned
7 and again assayed for binding specificity, in the SPRIA,
8 to the colon carcinoma extract but not to the normal
9 liver extract. This resulted in the generation of 29 CC
10 monoclonal antibodies which had binding specificity to
11 the colon carcinoma but not the normal liver extracts
12 (see Figure 1).

13 All of the 29 CC monoclonal antibodies shown
14 in Figure 1 exhibit binding specificity to extracts of
15 colon adenocarcinoma, but lack binding specificity to
16 extracts of the following normal and/or benign tissues:
17 colon (minimal binding specificity to superficial goblet
18 cells), ovary, stomach (minimal binding specificity to
19 goblet cells of intestinal metaplasia), endocervix
20 (minimal binding specificity to glandular epithelium),
21 brain, kidney, spleen, lung (minimal binding specificity
22 to epithelium), skin (minimal binding specificity to
23 sebaceous glandular epithelium), liver, prostate, uterus
24 (binding specificity to secretory phase endometrium
25 only), adrenal, pancreas, heart, lymph node, bone marrow,
26 breast and small intestine (minimal binding specificity
27 to superficial mucosal cells).

28 Of the 29 CC monoclonal antibodies so
29 produced, the hybridomas producing preferred monoclonal
30 antibodies have been deposited at the American Type
31 Culture Collection under CC 49 (ATCC No. HB-9459); CC 83
32 (ATCC No. HB-9453); CC 46 (ATCC No. HB-9458); CC 92 (ATCC
33 No. HB-9454); CC 30 (ATCC No. HB-9457); CC 11 (ATCC No.
34 HB-9455); and CC 15 (ATCC No. HB-9460).

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1 2. MATAG Group

2 The MATAG group was assayed in a SPRIA
3 essentially as described above for the CC group using a
4 1/80 dilution per well of TAG-72 in PBS except that to
5 detect binding of antibody, 50 μ l of rabbit-anti-mouse
6 IgM (Cooper Biomedical, Malvern, PA) was added to each
7 well. The plates were incubated for 1 hour at 37°C,
8 after which time 125 I-labelled Protein A (SPA)
9 (Pharmacia, Upsala, Sweden) at 50,000 cpm/25 μ l was added
10 per well and again allowed to incubate at 37°C for 1
11 hour. The unbound SPA was removed by extensive washing
12 with 1.0% (v/v) BSA in PBS.

13 Of the 2000 MATAG cultures assayed using
14 TAG-72 and PBS, 110 were found to have binding
15 specificity to TAG-72. Further cloning and assaying in a
16 SPRIA as described above, using TAG-72 yielded 34
17 cultures which had binding specificity with colon cancer
18 extract and TAG-72 but not a normal liver extract. These
19 were cloned into 3,264 wells and approximately 20 wells
20 of each of the original 34 cultures were assayed, in a
21 SPRIA as described above, using TAG-72 and PBS. This
22 yielded 23 cultures which had binding specificity to
23 TAG-72. The 23 cultures were subsequently grown up and
24 further assayed, in a SPRIA as described above, for lack
25 of binding specificity to normal spleen and normal liver
26 and binding specificity to a metastatic breast carcinoma
27 extract, as well as being assayed, in a SPRIA as
28 described above, using TAG-72 and PBS. The results
29 yielded 15 cultures which exhibited binding specificity
30 to the carcinoma extract and TAG-72 but not to the normal
31 extracts. These cultures were then recloned and
32 reassayed, in a SPRIA as described above, to produce 15
33 MATAG monoclonal antibodies (see Figure 1).

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1 All of the MATAG monoclonal antibodies shown
2 in Figure 1 exhibit binding specificity to extracts of
3 ovarian carcinoma, colon adenocarcinoma, infiltrating
4 ductal carcinoma of the breast, non-small cell lung
5 carcinoma, but lack binding specificity to extracts of
6 the following normal and/or benign tissues: colon
7 (minimal binding specificity to mucosal goblet cells),
8 ovary, benign effusions (minimal binding specificity to
9 lymphocytes and mesothelial cells), lung (minimal binding
10 specificity to bronchial epithelium), spleen, liver,
11 breast, kidney, bone marrow, stomach (minimal binding
12 specificity to superficial epithelium), skin, nerve,
13 parathyroid, heart, pancreas, lymph node, adrenal,
14 thyroid, small intestine (minimal binding specificity to
15 superficial mucosa), brain, gall bladder, cervix, uterus
16 (binding specificity to secretory phase of endometrium
17 only), endocervix (minimal binding specificity to
18 endocervical glandular epithelium), bladder, appendix,
19 fallopian tube, muscle, salivary gland, thymus, testis,
20 and esophagus.

21 Of the 15 MATAG monoclonal antibodies so
22 produced, the hybridoma producing MATAG 12 is preferred
23 and has been deposited at the American Type Culture
24 Collection under MATAG 12 (ATCC No. HB-9456).

25 Example 2

26 Isotyping Assay

27 1. CC Group

28 For the CC group, 50 µl of polyclonal anti-mouse
29 IgG (Jackson ImmunoResearch Laboratories, Inc., West
30 Grove, PA) was absorbed onto a 96-well polyvinyl chloride

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1 (Dynatech Laboratories, Alexandria, VA) microtiter
2 plate. The IgG was diluted with PBS. The plates were
3 incubated overnight at 37°C. The following day, 100 µl
4 of 5.0% (w/v) BSA in PBS was added to each well and
5 allowed to incubate for 1 hour to minimize non-specific
6 absorption. The wells were then washed with 1.0% (w/v)
7 BSA in PBS. 5 µl of undiluted CC culture supernatant was
8 added to each of two wells. The plates were again
9 incubated for 1 hour at 37°C after which time they were
10 washed 3 times with 1.0% (w/v) BSA in PBS. Rabbit-
11 anti-mouse IgG₁, IgG_{2b}, IgG₃, IgM, IgA (Cooper
12 Biomedical, Malvern, PA) DC-12 (NIH, NCI, LTIB) and
13 control 1.0% (w/v) BSA in PBS were added at 50 µl per
14 well. Following a 1 hour incubation, the plates were
15 washed 3 times as described above. Then 50,000 cpm of
16 ¹²⁵I-labelled Protein A (SPA) were added to each well,
17 incubated for 1 hour, washed 4 times with 1.0% (w/v) BSA
18 in PBS and the cpm per well was counted in a gamma
19 counter. The results are shown in Figure 1.

20 2. MATAG Group

21 For the MATAG group, isotypes were determined by
22 parallel assays essentially as described above for the CC
23 group. However, for detection, one assay used
24 ¹²⁵I-labelled goat-anti-mouse IgG (Kirkegaard & Perry,
25 Gaithersburg, MD) and the other assay used ¹²⁵I-labelled
26 goat-anti-mouse IgM (Kirkegaard & Perry, Gaithersburg,
27 MD) in place of ¹²⁵I-labelled Protein A (SPA).

28 The MATAG group was further characterized by High
29 Performance Liquid Chromatography (hereinafter "HPLC")
30 analysis for their pentameric structure. HPLC analysis
31 was performed using a Zorbax GF-450 column, 0.94 x 25 cm

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1 (Dupont, Wilmington, DE), equilibrated in 0.2 M sodium
2 phosphate (pH 6.8). 100 μ l MATAG culture supernatant was
3 loaded on the column and the column was run at a flow
4 rate of 0.5 ml/min, 0.5 ml fractions were collected at 1
5 min intervals. The fractions were analyzed for isotypes
6 as described above. The results are shown in Figure 1.

7 Example 3

8 Competition RIA

9 Competition RIAs were performed to determine
10 whether B72.3 and the CC monoclonal antibodies of the
11 present invention recognize different antigenic
12 determinants. More specifically, B72.3 and the CC
13 monoclonal antibodies were assayed for their ability to
14 compete for the binding of 125 I-labelled B72.3 to an
15 extract of LS-174T colon carcinoma cells in the following
16 manner.

17 5.0 μ g of LS-174T colon carcinoma cell extract was
18 absorbed in each well of a polyvinyl chloride microtiter
19 plate (Dynatech Laboratories, Alexandria, VA) and varying
20 amounts of competing CC monoclonal antibody (from 10
21 μ g/ μ l to 0.004 μ g/ μ l) was added to saturate the binding
22 sites. After incubation for 6 hours at 4°C, 50,000
23 cpm/25 μ l of 125 I-B72.3, was added to each well and
24 incubated for 12 hours at 4°C. Bound 125 I-B72.3 was
25 determined by cutting individual wells and measuring cpm
26 in the wells in a gamma counter. The cpm in the wells
27 pre-incubated with saturating amounts of B72.3 as a
28 competitor was considered 100% competition. The results
29 are shown in Figure 2A, 2C, 2E, and 2G. In Figure 2A, CC
30 41 was used as the competing antibody. In Figure 2C, CC
31 60 was used as the competing antibody. In Figure 2E,

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1 CC 83 was used as the competing antibody. In Figure 2G,
2 CC 49 was used as the competing antibody.

3 As shown in Figures 2A and 2C, CC 41 and CC 60 did
4 not compete at all with B72.3. This demonstrates that CC
5 41 and CC 60 having specificity for a different epitope
6 on TAG-72 than B72.3. As shown in Figures 2E and 2G, CC
7 83 and CC 49 partially compete with B72.3. This
8 demonstrates that the epitopes recognized by CC 83 and CC
9 49 share partial (but not complete) homology with the
10 B72.3 epitope on the TAG-72 molecule, or that the CC 83
11 and CC 49 epitopes are distinct from but near the B72.3
12 epitope, resulting in steric hinderance.

13 Thereafter, competition RIAs were performed to
14 determine whether CC 49 recognizes the same or different
15 antigenic determinants than B72.3, CC 30, CC 46, and CC
16 83. More specifically, these monoclonal antibodies were
17 assayed for their ability to compete for the binding of
18 ^{125}I -labelled CC 49 to an extract of LS-174T colon
19 carcinoma cells as described above. The results obtained
20 are shown in Figure 3. Figure 3 demonstrates that (1)
21 the epitopes on TAG-72 recognized by monoclonal
22 antibodies CC 46 and B72.3 share little or no homology
23 with the epitope recognized by monoclonal antibody CC 49;
24 (2) the epitope recognized by CC 83 shares considerable
25 homology with that recognized by CC 49 but is not
26 identical as revealed by the displacement of the CC 83
27 curve; and (3) the epitope recognized by monoclonal
28 antibody CC 30, shares partial homology to that
29 recognized by CC 49, or is distinct from that of CC 49
30 but is in proximal location resulting in steric
31 hinderance.

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1 Example 4
2 Binding Affinity

3 The binding affinities (affinity constants) of the
4 second generation monoclonal antibodies of the present
5 invention to TAG-72 were determined by a SPRIA using a
6 modification of the procedure of Heyman, et al., J.
7 Immunol. Methods, 68:193-204 (1984). More specifically,
8 30 μ l of purified TAG-72 diluted in PBS at a
9 concentration of 280 units/ml (units determined as
10 described in Paterson, et al., Intl. J. Cancer, 37:659-
11 666 (1986)) were dried in 96 well polyvinyl chloride
12 microtiter plates (Dynatech Laboratories, Alexandria,
13 VA). Any remaining non-specific active groups were
14 blocked with 5.0% (v/v) BSA in PBS. Then, 20 μ l of 1:1.5
15 serial dilutions of the purified monoclonal antibody
16 (purified as described in Colcher, et al., Cancer Res.,
17 44:5744-5751 (1984)), shown in Table 2 below, starting at
18 1.0 μ g/ml were added to the wells. After incubating
19 overnight at 4°C, the plates were washed three times with
20 1.0% (v/v) BSA in PBS. Next, ¹²⁵I-labelled
21 goat-anti-mouse IgG (Kirkegaard & Perry, Gaithersburg,
22 MD) at 75,000 cpm/25 μ l per well was added and left to
23 react for 1 hour at 37°C. After washing three times with
24 1.0% (v/v) BSA in PBS, the cpm in the individual wells
25 were counted as described above.

26 In order to convert the cpm values to
27 concentration of bound monoclonal antibody, the remaining
28 free monoclonal antibodies in the supernatant, which had
29 been incubated with TAG-72 but not bound thereto, were
30 incubated on another 96 well polyvinyl chloride
31 microtiter plate which had been precoated with 4.0 g/ml
32 of sheep anti-mouse IgG (Jackson Immunoresearch
33 Laboratories, Inc., West Grove, PA) and detected with

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1 ^{125}I -labelled goat anti-mouse IgG (Kirkegaard & Perry,
2 Gaithersburg, MD). In this manner, the concentration at
3 which there was no free monoclonal antibodies remaining
4 in the supernatant was determined for each monoclonal
5 antibody. From these data, computer curves were
6 generated to determine the binding affinity constant of
7 each monoclonal antibody. The results are shown in Table
8 2 below.

9 TABLE 2

10 Binding Affinity Constants
11 Measured Using TAG-72

12		Affinity Constant
13	<u>Purified Antibody</u>	<u>($\times 10^9\text{M}$)</u>
14	B72.3	2.54
15	CC 46	3.64
16	CC 30	8.15
17	CC 15	9.13
18	CC 29	9.49
19	CC 92	14.26
20	CC 49	20.58
21	CC 83	27.72

22 Table 2 demonstrates that the second generation
23 monoclonal antibodies CC 46, CC 30, CC 15, CC 29, CC 92,
24 CC 49 and CC 83 all have higher binding affinity
25 constants than the first generation monoclonal antibody
26 B72.3.

27 The CC group was assayed in a SPRIA using the cell
28 extracts from the LS-174T cell line and a metastatic
29 breast carcinoma. 50 μl of the cell extract (5 μg) was

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1 added to each well of a Cooke round bottom polyvinyl
2 chloride microtiter plate (Dynatech Laboratories,
3 Alexandria, VA) and allowed to dry. To minimize
4 non-specific protein absorption, microtiter wells were
5 treated with 100 μ l of 5.0% (v/v) BSA in PBS and
6 incubated covered for 1 hour. This and all subsequent
7 incubations were at 37°C. The BSA was then removed and
8 the wells were washed one time with 1.0% (v/v) BSA in
9 PBS. Next, 50 μ l of hybridoma supernatant and 1:5
10 dilutions of the supernatant fluid was added per well.
11 After a 1 hour incubation, the unbound immunoglobulin was
12 removed by washing the plates three times with 1.0% (v/v)
13 BSA in PBS at 100 μ l/well/wash.

14 To determine antibody binding, the wells were then
15 incubated with 25 μ l of 125 I-goat-anti-mouse IgG (gamma
16 chain specific) (Kirkegaard & Perry, Gaithersburg, MD) at
17 75,000 cpm/25 μ l per well for 1 hour at 37°C. The
18 supernatant was aspirated and the plates were washed four
19 times with 1.0% (v/v) BSA in PBS at 100 μ l/well/wash.
20 The bound cpm were detected by cutting the individual
21 wells from the plate and measuring the CPM in a gamma
22 counter.

23 As shown in Figure 2B, CC 41 reacts with the LS
24 extract but B72.3 does not. Note, Figure 2B and Table 2
25 demonstrates that CC 41 has a higher binding affinity
26 (slope of the curve) to the Br. Ca. than B72.3. Figure
27 2D demonstrates that although CC 60 does not have binding
28 specificity to the LS extract like B72.3, CC 60 has a
29 higher binding affinity (slope of the curve) to the Br.
30 Ca. than B72.3. Figure 2F demonstrates that CC 83 and
31 B72.3 have similar binding properties to the Br. Ca.
32 extract but that CC 83 has high binding affinity to the
33 LS extract while B72.3 does not. Figure 2H demonstrates

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1 that CC 49 has high binding affinity to both the LS and
2 Br. Ca. extracts while B72.3 has essentially no binding
3 affinity to the LS extract.

4 Example 5
5 Western Blotting

6 40 µg of LS-174T cell extracts or an extract of a
7 human breast carcinoma diluted in SDS-PAGE sample buffer
8 comprising 0.125 M Tris-HCl (pH 6.8) 4.0% (w/v) SDS, 20%
9 (v/v) glycerol and 10% (v/v) 2-mercaptoethanol, loaded
10 onto a 3 to 12% (v/v) linear gradient SDS-PAGE. After
11 electrophoresis for 8 hours at 5 milliamps/gel at 9°C,
12 the gels were treated with transfer buffer comprising 25
13 mM Tris-HCl (pH 8.3), 192 mM glycine, 20% (v/v) methanol
14 with 4 M urea and 0.5% Triton-X-100 for 1 hour at room
15 temperature. The gel was then equilibrated with
16 transfer buffer and the proteins were transferred to
17 nitrocellulose paper (0.45 µm pore size) at 4°C for 16
18 hours at 30 V. Then, the nitrocellulose paper was
19 incubated with 5.0% (w/v) BSA with 0.05% (v/v) Tween-20
20 in PBS for 3 hours at room temperature and washed with
21 0.05% (v/v) Tween-20 in PBS. Next, 10 ml of hybridoma
22 tissue culture supernatant of all the CC and MATAG
23 monoclonal antibodies were added, and incubation
24 continued for 2 hours at room temperature with gentle
25 agitation. After washing with PBS containing 0.05% (v/v)
26 Tween-20, the nitrocellulose paper was incubated for 1
27 hour at room temperature with ¹²⁵I-labelled
28 goat-anti-mouse IgG (Kirkegaard & Perry, Gaithersburg,
29 MD). The nitrocellulose paper was then extensively
30 washed overnight and exposed to Kodak XAR-5 X-ray film
31 with a DuPont Lightning Plus intensifying screen at

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1 -70°C for 2 hours. For all experiments, NS-1 tissue
2 culture supernatant was used as a negative control.

3 The Western blotting analysis demonstrated the
4 reactivity of the CC and MATAG antibodies to a diffuse
5 band beginning at the interface of the stacking gel with
6 the 5-12% resolving gel that penetrated the resolving gel
7 approximately 1 cm. This diffuse band is consistent with
8 the high molecular weight TAG-72 mucin-like molecule.
9 The high molecular weight band was observed with all the
10 CC and MATAG antibodies tested and detected in both the
11 LS-174T cell line extract and the human breast carcinoma
12 metastases extract.

13 Example 6

14 Immunoperoxidase Studies

15 5.0 μ sections of formalin-fixed or frozen
16 sections of tissue on slides were used. Fixed tissues
17 were deparaffinized in xylene and hydrated in graded
18 H₂O/ethanol rinses. A 15 minute incubation with 0.3%
19 (v/v) H₂O₂ in methanol was used to block any endogenous
20 peroxidase activity. After rinsing in PBS without Ca⁺²
21 and Mg⁺², the slides were incubated with a 1:10 (v/v)
22 dilution of normal goat serum for the MATAG designated
23 antibodies for 15 minutes. This incubation and all
24 subsequent incubations were carried out at room
25 temperature with the exception of the primary MATAG
26 antibody which was a 16 hour incubation at 4°C. The
27 normal blocking serum was removed and undiluted tissue
28 culture supernatant of the monoclonal antibody was placed
29 on the tissue sections and the slides were incubated
30 overnight. The supernatant IgM was

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1 removed and the slides were rinsed for 15 minutes in PBS
2 without Ca^{+2} and Mg^{+2} . For the MATAG designated
3 antibodies at 1:167 (v/v) dilution of biotinylated goat
4 anti-murine IgM (Vector Laboratories, Inc.), was added to
5 each of the tissue sections and allowed to incubated for
6 30 minutes. The slides were again rinsed in PBS without
7 Ca^{+2} and Mg^{+2} and then incubated for 30 minutes with ABC
8 (Vector Laboratories, Inc.) peroxidase at room
9 temperature. After another PBS rinse, 0.06% (v/v) 3,3'
10 diaminobenzidine (Sigma Chemical Co., St. Louis, MO) with
11 0.01% (v/v) H_2O_2 was added for 5 minutes. The sections
12 were rinsed briefly in water, counterstained with
13 hematoxylin, dehydrated in graded ethanol/ H_2O rinses,
14 cleared (eliminating residual H_2O) in xylene, mounted
15 with Permount (histologic mounting medium, Fisher
16 Scientific Co.) under a coverslip, and examined with a
17 light microscope. Each section was evaluated for the
18 presence of reddish-brown diaminobenzidine precipitate
19 indicative of monoclonal antibody binding. The
20 approximate percentage of positive carcinoma cells was
21 assigned according to the number of carcinoma cells
22 positive with each monoclonal antibody divided by the
23 total number of carcinoma cells times 100. The results
24 are shown in Table 3 below.

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TABLE 3

Binding Specificity of B72.3 vs. MATAG-12 in
an Immunoperoxidase Assay of Tissue Sections

<u>Percent MAb Reactive Carcinoma Cells</u>			
	<u>B72.3</u>	<u>MATAG-12</u>	
Ovarian Cancer 1	6	80	
Ovarian Cancer 2	5	25	
Ovarian Cancer 3	10	55	
Colorectal Cancer 1	10	60	
Colorectal Cancer 2	40	95	

As shown in Table 3, the percent carcinoma cells reactive with B72.3 is considerably lower than that for MATAG-12. This demonstrates that MATAG-12 has a higher binding specificity for the above carcinomas and thus is more useful in immunohistochemical or immunocytochemical assays, as well as in in vivo diagnosis and therapy of cancer.

Example 7In Vivo Carcinoma Testing

The monoclonal antibodies shown in Table 4 below were labelled with Na¹²⁵I using Iodogen (Pierce Chemical, Rockford, IL). More specifically, 40 µg of monoclonal antibody shown in Table 4 below were adjusted 0.1 ml 0.1 M sodium phosphate buffer (pH 7.2) and then added to a 12 cm x 75 cm glass tube coated with 20 µg of Iodogen followed by addition of 0.5 mCi of Na¹²⁵I (New England Nuclear, Boston, MA). After a 2 min

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1 incubation at room temperature, the protein was removed
2 from the insoluble Iodogen, and the unincorporated ^{125}I
3 was separated from the antibody by gel filtration through
4 a 10 ml column Sephadex G-25 with a buffer comprising 10
5 mM sodium phosphate, pH 7.2. The labelled monoclonal
6 antibody in the void was pooled and dialyzed against 10
7 mM sodium phosphate buffer (pH 7.2) containing 5.0 mM
8 NaI. The iodination protocol yielded labelled IgG
9 monoclonal antibody with a specific activity of 5.0 to 15
10 $\mu\text{Ci}/\mu\text{g}$ (approximately 8.0 to 25×10^6 cpm/ μg).

11 Female athymic mice (nu/nu) on a BALB/c background
12 were obtained from Charles River, Inc., or the Frederick
13 Cancer Research Facility at approximately 4 weeks of
14 age. One week later, mice were inoculated subcutaneously
15 (0.1 ml/mouse) with the LS-174T human colon carcinoma
16 cells (1×10^6 cells/animal).

17 Athymic mice bearing carcinomas 0.3 to 1.5 cm in
18 diameter, approximately 2 to 3 weeks after inoculation of
19 the cells were given injections intraperitoneally of 1.5
20 μCi (0.1 μg) in PBS of the monoclonal antibodies shown in
21 Table 4 below, which had been iodinated as described
22 above. Groups of five mice were sacrificed at varying
23 times by exsanguination, the carcinoma and normal tissues
24 were excised and weighed, and the cpm were measured in a
25 gamma counter. The cpm/mg of each tissues was then
26 determined and compared to that found in the carcinoma.
27 The results are shown in Table 4 and Figures 4A and 4B.

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TABLE 4
Percent Injected Dose Per Gram of
 ^{125}I -Labelled Antibody*

Tissue	B72.3	CC 11	CC 46	CC 30	CC 92	CC 83	CC 49
Carcinoma	6.6	26.6	13.2	23.1	12.4	22.9	23.4
Liver	0.8	1.2	0.5	0.8	0.8	0.7	1.2
Spleen	0.5	1.1	0.5	1.0	1.0	0.7	1.2
Kidney	0.6	1.1	0.4	1.0	1.0	0.7	0.4
Lung	1.4	2.4	1.1	2.1	2.0	1.8	0.6
Blood	2.9	6.2	2.1	4.1	3.8	4.6	1.1

*At 168 hours post monoclonal antibody administration.

As shown in Table 4, the percent of injected dose to tumor for B72.3 is considerably lower than that for the CC antibodies of the present invention. Even though monoclonal antibody CC 46 has only a slightly higher affinity constant than B72.3, Table 4 shows that CC 46 is clearly more efficient in targeting the human tumor in situ than is B72.3. This demonstrates that the second generation monoclonal antibodies of the present invention are more efficient for in vivo carcinoma targeting than monoclonal antibody B72.3 and thus are more useful in in vivo diagnosis and therapy of cancer. Figures 4A and 4B show the different binding kinetics and carcinoma/normal tissue ratios at various time points for CC 11 and CC 46, respectively. Figures 4A and 4B demonstrate that these monoclonal antibodies have the ability to bind the carcinomas efficiently and stay bound to the carcinomas over a prolonged time (i.e., at least 7 days).

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Example 8Fragmentation of Monoclonal Antibodies

Biodistribution studies both in animal models and in clinical trials have demonstrated that intact IgG may not be the best form of the antibody molecule to obtain optimal tumor localization with minimal background in normal organs. As a result, studies were undertaken to fragment the second generation monoclonal antibodies of the present invention and B72.3 with pepsin as described in Colcher, et al., Cancer Res., 43:736-742 (1983). The resulting fragments were radiolabelled with ^{125}I as described above and tested for binding specificity in a SPRIA as described above, using a LS-174T colon carcinoma cell extract. The results are shown in Table 5.

TABLE 5

Binding Specificity of Immunoreactive
F(ab')₂ Fragments

	<u>F(ab')₂ Fragment</u>	<u>Binding Specificity to LS-174T colon carcinoma cell extract</u>
20	B72.3	<2%
21	CC 49	50%
22	CC 46	70%

As shown in Table 5, F(ab')₂ fragments of CC 49 were able to bind greater than 50% of the input counts in a SPRIA using limiting amounts of antigen and CC 46 fragments bound over 70% of the input activity while fragments obtained from B72.3 essentially lack all immunoreactivity, i.e., maintained less than 2% binding specificity.

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1 A pharmaceutical composition comprising the second
2 generation antibodies of the present invention in a
3 pharmaceutically acceptable, non-toxic, sterile carrier
4 such as physiological saline, non-toxic buffers and the
5 like, now also becomes possible. The amount of said
6 antibodies in the pharmaceutical composition should be
7 sufficient to achieve effective binding with the antigens
8 against which said antibodies have specific affinity or
9 neutralization reactivity. The pharmaceutical
10 composition may be administered in a single or multiple
11 dosage with other adjuvants or additives, if necessary,
12 in any suitable manner to the host in need of said
13 antibodies.

14 While this invention has been described in detail
15 and with reference to specific embodiments thereof, it
16 will be apparent to one skilled in the art that various
17 changes and modifications could be made therein without
18 departing from the spirit and scope thereof.

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1 WHAT IS CLAIMED IS:

2 1. A second generation monoclonal antibody,
3 immunoreactive fragment or recombinant thereof, having
4 binding affinity for both TAG-72 and LS-174T cell line
5 antigen without substantial binding affinity for normal
6 adult human tissues.

7 2. The second generation monoclonal antibody of
8 Claim 1, wherein said antibody has a binding affinity of
9 greater than 3×10^9 M.

10 3. The second generation monoclonal antibody of
11 Claim 1, wherein said antibody has about 50% more
12 efficiency than B72.3 antibody in targeting human
13 carcinomas in situ.

14 4. The second generation monoclonal antibody of
15 Claim 1, wherein said antibody exhibits 0-30% competition
16 with B72.3.

17 5. The second generation monoclonal antibody of
18 Claim 1, wherein said antibody is of an isotype selected
19 from the group consisting of IgG_{2a}, IgG_{2b}, IgG₃, and IgM.

20 6. The second generation antibody of Claim 1,
21 wherein said antibody is conjugated to a label, a tumor
22 detecting marker or to a therapeutic agent.

23 7. The second generation antibody of Claim 6,
24 wherein said label is selected from the group consisting
25 of a radioisotope, a fluorescent molecule and an enzyme.

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1 8. The second generation antibody of Claim 7,
2 wherein said radioisotope is selected from the group
3 consisting of ^{32}P , ^{14}C , ^3H , ^{125}I , and ^{35}S .

4 9. The second generation antibody of Claim 7,
5 wherein said fluorescent molecule is selected from the
6 group consisting of fluorescein and rhodamine.

7 10. The second generation antibody of Claim 7,
8 wherein said enzyme is selected from the group consisting
9 of alkaline phosphatase and horseradish peroxidase.

10 11. The second generation antibody of Claim 6,
11 wherein said tumor detecting marker is selected from the
12 group consisting of ^{131}I , ^{123}I , ^{111}In , ^{67}Ga , $^{99\text{m}}\text{Tc}$ and
13 Gd.

14 12. The second generation antibody of Claim 6,
15 wherein said therapeutic agent is selected from the group
16 consisting of a radionuclide, drug, toxin and second
17 antibody.

18 13. The second generation antibody of Claim 12,
19 wherein said radionuclide is selected from the group
20 consisting of ^{131}I , ^{90}Y , ^{105}Rh , ^{47}Sc , ^{67}Cu , ^{212}Bi , and
21 ^{211}At .

22 14. The second generation antibody of Claim 12,
23 wherein said drug is selected from the group consisting
24 of methotrexate and adriamycin.

25 15. The second generation antibody of Claim 23,
26 wherein said second antibody has specific binding
27 affinity to killer T-cells.

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1 16. The second generation antibody of Claim 1,
2 obtained from a hybridoma selected from the group
3 consisting of the hybridomas having the identifying
4 characteristics of ATCC No. HB-9459, ATCC No. HB-9453,
5 ATCC No. HB-9458, ATCC No. HB-9454, ATCC No. HB-9457,
6 ATCC No. HB-9455, ATCC No. HB-9460, and ATCC No. HB-9456.

7 17. A method for detecting a human carcinoma or
8 metastases thereof comprising:

9 (a) obtaining a sample of body fluid or
10 biopsy from a patient;

11 (b) contacting the body fluid or biopsy
12 with the second generation monoclonal antibody,
13 immunoreactive fragment or recombinant thereof of Claim
14 1;

15 (c) determining the amount of binding of
16 second generation monoclonal antibody, immunoreactive
17 fragment or recombinant thereof to the body fluid or
18 biopsy material; and

19 (d) comparing the amount of binding in step
20 (c) to a control sample or to a predetermined base level;
21 a binding greater than the base level being indicative of
22 the presence of carcinomas or metastases thereof.

23 18. The method of Claim 17, wherein said body
24 fluid is selected from the group consisting of blood,
25 plasma, serum, nipple discharge, cyst fluid, ascites
26 fluids, pleural effusions, seminal plasma, semen, urine
27 and prostatic fluid.

28 19. The method of Claim 17, wherein the amount
29 of monoclonal antibody binding to material present in the
30 body fluid or biopsy is determined by means of a
31 radioimmunoassay.

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1 20. The method of Claim 17, wherein the amount
2 of monoclonal antibody binding to substances present in
3 the body fluid or biopsy is determined by means of an
4 enzyme immunoassay.

5 21. The method of Claim 17, wherein said
6 antibody has a binding affinity of greater than 3×10^9
7 M.

8 22. The method of Claim 17, wherein said
9 antibody exhibits 0-30% competition with B72.3 antibody.

10 23. The method of Claim 17, wherein said
11 antibody is of an isotype selected from the group
12 consisting of IgG_{2a}, IgG_{2b}, IgG₃, and IgM.

13 24. The method of Claim 17, wherein said
14 antibody is obtained from a hybridoma selected from the
15 group consisting of hybridomas having the identifying
16 characteristics of ATCC No. HB-9459, ATCC No. HB-9453,
17 ATCC No. HB-9458, ATCC No. HB-9454, ATCC No. HB-9457,
18 ATCC No. HB-9455, ATCC No. HB-9460, and ATCC No. HB-9456.

19 25. A method for localizing carcinoma or
20 metastases thereof comprising:

21 (a) administering to a patient a second
22 generation monoclonal antibody, immunoreactive fragment
23 or recombinant thereof of Claim 1, conjugated to an
24 imaging or detecting marker; and

25 (b) exposing a patient to means for
26 detecting said tumor detecting marker, an area of
27 localization of the tumor detecting marker being
28 indicative of the site of the carcinoma or metastasis in
29 said patient.

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1 26. The method of Claim 25, wherein said tumor
2 detecting marker is selected from the group consisting of
3 ^{125}I , ^{131}I , ^{123}I , ^{111}In , ^{113}In , ^{67}Ga , ^{68}Ga , $^{99\text{m}}\text{Tc}$ and Gd.

4 27. The method of Claim 25, wherein said
5 antibody has a binding affinity of greater than 3×10^9
6 M.

7 28. The method of Claim 25, wherein said
8 antibody exhibits 50% more efficiency than B72.3 in
9 targeting human carcinoma in situ.

10 29. The method of Claim 25, wherein said
11 antibody exhibits 0-30% competition with B72.3 antibody.

12 30. The method of Claim 25, wherein said
13 antibody is of an isotype selected from the group
14 consisting of IgG_{2a}, IgG_{2b}, IgG₃, and IgM.

15 31. The method of Claim 25, wherein said
16 antibody is obtained from a hybridoma selected from the
17 group consisting of hybridomas having the identifying
18 characteristics of ATCC No. HB-9459, ATCC No. HB-9453,
19 ATCC No. HB-9458, ATCC No. HB-9454, ATCC No. HB-9457,
20 ATCC No. HB-9455, ATCC No. HB-9460, and ATCC No. HB-9456.

21 32. A method for treating carcinoma or
22 metastases thereof, comprising administering to a patient
23 afflicted with carcinoma or metastases thereof, an
24 effective amount of a second generation monoclonal
25 antibody, immunoreactive fragment or recombinant thereof
26 of Claim 1 to destroy or inhibit growth and proliferation
27 of said carcinoma or metastases thereof.

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1 33. The method of Claim 32, wherein said
2 antibody is conjugated to a therapeutic agent.

3 34. The method as claimed in Claim 33, wherein
4 said therapeutic agent is selected from the group
5 consisting of a radionuclide, a drug, a toxin, a
6 biological response modifier, and a second antibody.

7 35. The method of Claim 34, wherein said
8 radionuclide is selected from the group consisting of
9 ^{131}I , ^{90}Y , ^{105}Rh , ^{47}Sc , ^{67}Cu , ^{212}Bi , and ^{211}At .

10 36. The method of Claim 34, wherein said drug is
11 selected from the group consisting of methotrexate and
12 adriamycin.

13 37. The method of Claim 34, wherein said second
14 antibody has specific binding affinity to killer T-cells.

15 38. The method of Claim 32, wherein said
16 antibody has a binding affinity of greater than 3×10^9
17 M.

18 39. The method of Claim 32, wherein said
19 antibody exhibits 50% or more efficiency than B72.3 in
20 targeting human carcinoma in situ.

21 40. The method of Claim 32, wherein said
22 antibody exhibits 0-30% competition with B72.3 antibody.

23 41. The method as claimed in Claim 32, wherein
24 said antibody is of an isotype selected from the group
25 consisting of IgG_{2a} , IgG_{2b} , IgG_3 and IgM .

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1 42. The method of Claim 32, wherein said
2 antibody is obtained from a hybridoma selected from the
3 group consisting of hybridomas having the identifying
4 characteristics of ATCC No. HB-9459, ATCC No. HB-9453,
5 ATCC No. HB-9458, ATCC No. HB-9454, ATCC No. HB-9457,
6 ATCC No. HB-9455, ATCC No. HB-9460, and ATCC No. HB-9456.

7 43. A hybridoma producing a second generation
8 monoclonal antibody having specific binding affinity to
9 both TAG-72 and LS-174T antigens without substantial
10 binding affinity to normal adult human tissues.

11 44. The hybridoma of Claim 43, selected from the
12 group consisting of hybridomas having the identifying
13 characteristics of ATCC No. HB-9459, ATCC No. HB-9453,
14 ATCC No. HB-9458, ATCC No. HB-9454, ATCC No. HB-9457,
15 ATCC No. HB-9455, ATCC No. HB-9460, and ATCC No. HB-9456.

16 45. A pharmaceutical composition comprising the
17 antibody, immunoreactive fragment or recombinant thereof,
18 of Claim 1 in an amount sufficient to bind antigens for
19 which said antibody or part thereof has specific binding
20 affinity and pharmaceutically acceptable, non-toxic,
21 sterile carrier.

22 46. A composition of matter comprising an
23 immuogen which is an antibody of Claim 1.

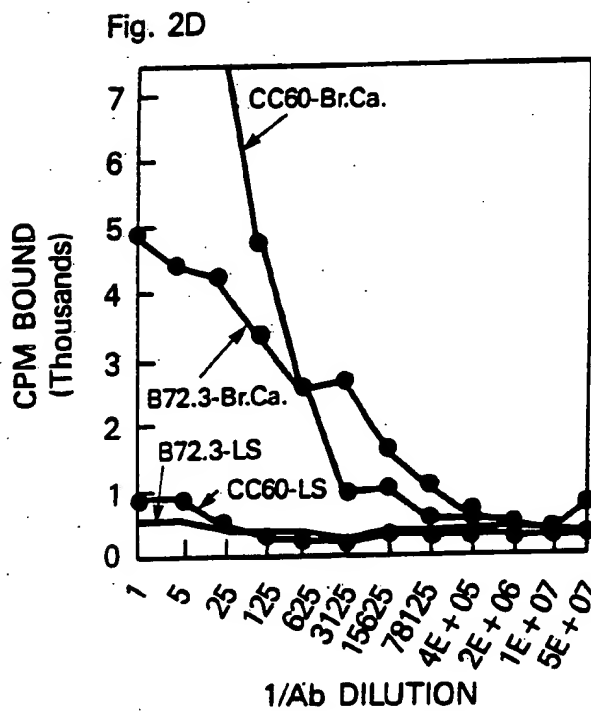
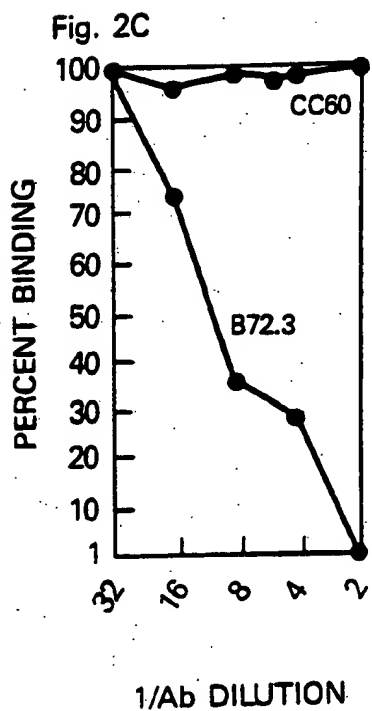
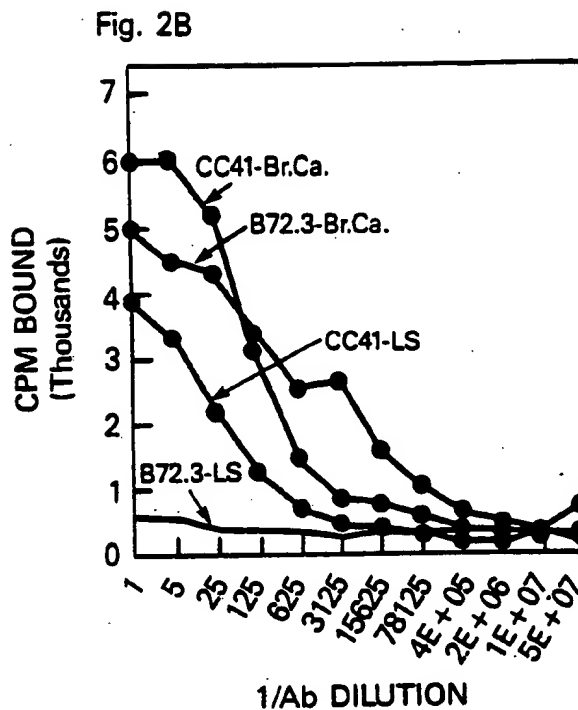
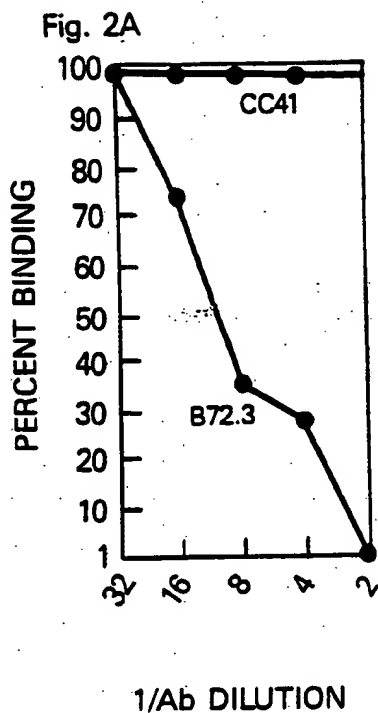
24 47. A method of producing anti-idiotypic
25 antibodies by administration of an immunogenic effective
26 amount of a composition of Claim 46 to a mammal.

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1 48. A method of eliciting antibodies against a
2 tumor of a cancer patient by administration of a
3 composition of Claim 46.

% Competition in B72.3 RIA	No.MAb	Isotype	No.MAb	Binding Affinity to Colon Carcinoma	No.MAb	MAB Designation
0 - 30%	9	G ₁	7	6/8	5	CC 29,41,66,87,92
		G _{2a}	1	5/8	2	CC 50,52
		G _{2b}	1	5/8	1	CC 60
		M	0	6/8	1	CC 15
31 - 70%	19	G ₁	14	6/8	12	CC 1,8,14,20,28,30, 49,55,57,78,83,90
		G _{2a}	0	5/8	2	CC 46,72
		G _{2b}	1	6/8	1	CC 48
		M	4	5/6	3	MATAG 9,11,18
71 - 100%	16	G ₁	5	4/6	1	MATAG 10
		G _{2a}	0	6/8	5	CC 9,11,40,63,74
		G _{2b}	0	5/6	9	MATAG 1,2,3,4,7,12 14,15,17
		M	11	4/6	2	MATAG 5,6
Total MABs						

Figure 1



Ab DILUTION (1 : 5)

Fig. 2E

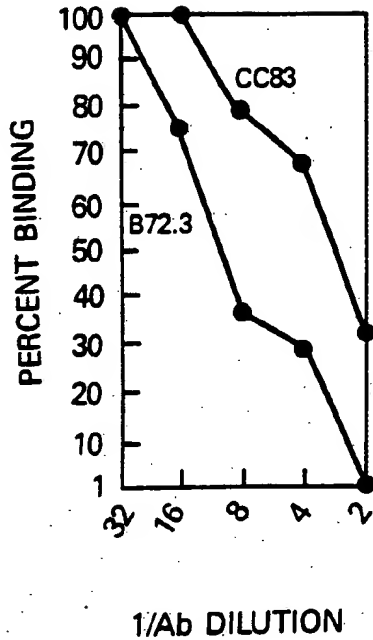


Fig. 2F

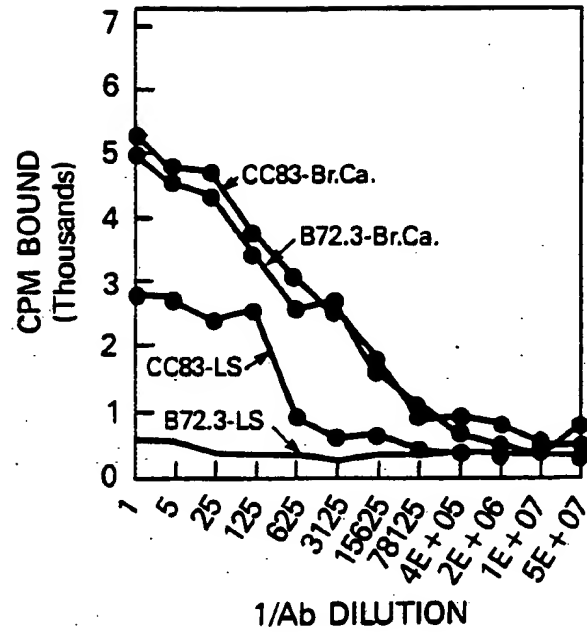


Fig. 2G

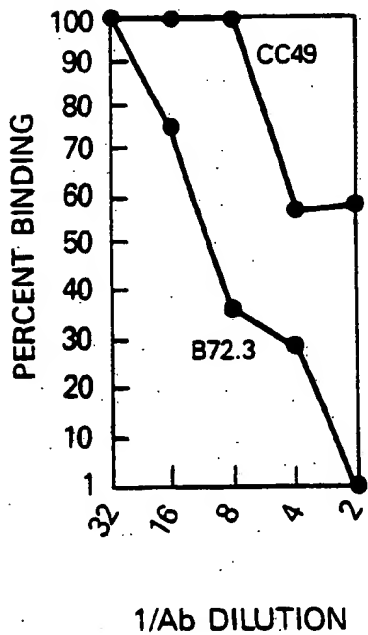
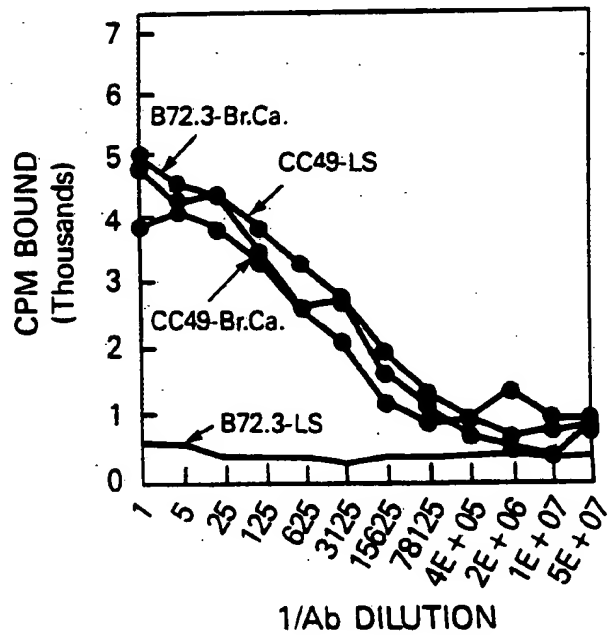


Fig. 2H



Ab DILUTION (1 : 5)

Figure 3

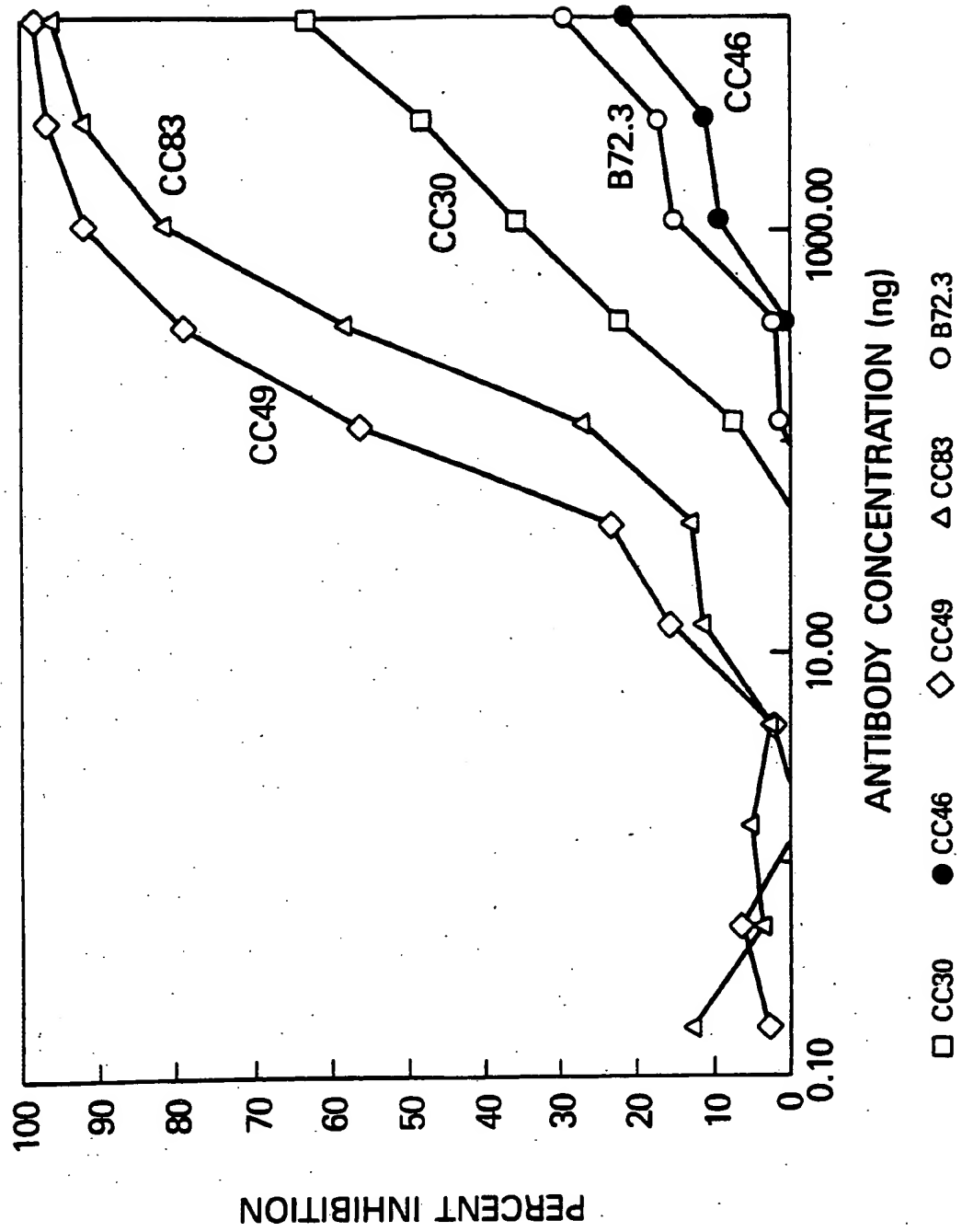


Fig. 4A

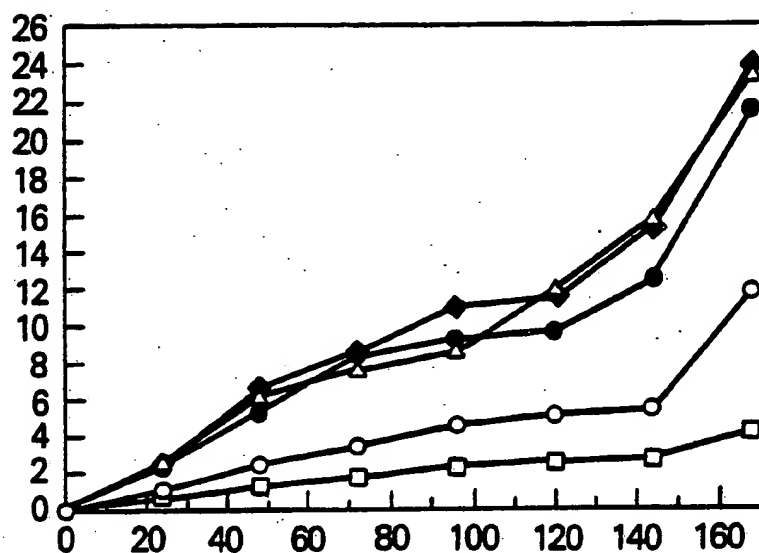
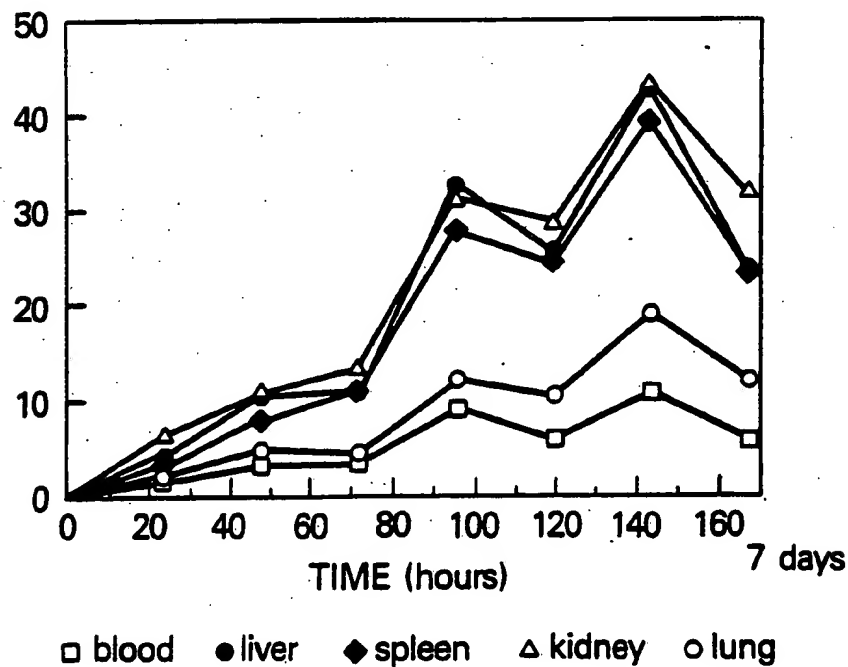


Fig. 4B



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/01941

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (4): G01N 33/53		
U.S. CL.: 435/7		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
U.S.	435/7; 436/63, 64, 512, 548, 813	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
Chemical Abstracts Services Online (File CA, 1967-1988; File Biosis Previews 1969-1988). Automated Patent System (File USPAT, 1975-1988).		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X Y	Cancer, Volume 57, No. 3, published 1986 (Philadelphia, Pennsylvania, U.S.A.), J. Lundy, "Radioimmuno-detection of Human Colon Carcinoma Xenografts in Visceral Organs of Congenitally Athymic Mice", see Abstract.	1, 25, 26 27-31
X Y	Biological Abstracts, Volume 81, No. 2, issued 15 January 1986, (Philadelphia, Pennsylvania, USA), S.C. Lottich, "Tumor-associated Antigen TAG-72: Correlation of Expression in Primary and Metastatic Breast Cancer Lesions", see page 679, column 1, the abstract No. 15748, Breast Cancer Res. Treat. 1985, 6(1), 49-56 (Eng).	1, 17, 18, 20 19, 21-24
<p>[*] Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
01 November 1988	05 DEC 1988	
International Searching Authority	Signature of Authorized Officer	
ISA/US	TONI R. SCHEINER	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
<u>X</u> Y	Biological Abstracts, Volume 82, No. 3, issued 1 August 1986 (Philadelphia, Pennsylvania, USA), A.J. Paterson, "A Radioimmunoassay for the Detection of a Tumor-associated Glycoprotein (TAG-72) using Monoclonal Antibody B72.3," see page 685, column 1, the abstract No. 36028, Int. J. Cancer 1986, 37(5), 659-666 (Eng).	<u>1,17-19</u> <u>2-8,11,</u> 20-24
Y	Clin. Chem., Volume 27, No. 11, issued 1981 (Winston-Salem, North Carolina, USA), E.D. Sevier, "Monoclonal Antibodies in Clinical Immunology", 1797-1806, see page 1799, column 2, 1st paragraph, page 1800, column 1, last paragraph, and column 2, 1st paragraph.	1-46
Y	JNCI, Volume 76, No. 6, issued June 1986 (Washington, D.C. USA), F. Gorstein, "Tumor-Associated Glycoprotein (TAG-72) in Ovarian Carcinomas Defined by Monoclonal Antibody B72.3", 995-1003, See Abstract.	1-48
Y	R. LEVY, "Monoclonal Antibodies in Approaches to Tumor Immunology", Summary of Minisymposium presented by the American Association of Immunologists at the 66th Annual Meeting of the Federation of American Societies for Experimental Biology, New Orleans, Louisiana, USA, 16 April 1982, "Tumor therapy with Monoclonal Antibodies", see pages 2655-2656, Future Prospects.	32-48
<u>X</u> <u>Y</u>	N. Ohuchi, "In vivo Application of Monoclonal Antibodies in the Management of Human Carcinomas", Medline, Index Medicus, Gan To Kagaku Ryoho (Japan) April 1988, Volume 15, No. 4, 1109-1114, see the abstract.	<u>1,25,26</u> <u>27-31</u>
Y	H. HEDIN, "Tumor Localization of CEA Containing Human Tumors in Nude Mice by Means of Monoclonal Anti-CEA Antibodies", Medline, Index Medicus, Int. J. Cancer, 15 November 1982, Volume 30, No. 5, 547-552, see the abstract.	25-31

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	US, A, 4,361,544 (Goldenberg) 30 November 1982, see Abstract, column 13, lines 45-62, column 14 lines 48-68 and column 15, lines 1-7.	17-48
Y	US, A, 4,634,586 (Goodwin) 6 January 1987, see Abstract and column 8, lines 5-21.	17-31

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

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